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Chemical Studies into the Amino Acids Present in Latent Fingermarks

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

Date: **27/01/2015**

Abstract

Fingermarks are a vital component of forensic investigations to link a suspect to a crime scene. Being able to visualise the ridge detail of latent (invisible) fingermarks is therefore of utmost importance for law enforcement agencies. Novel reagents may offer alternative methods for the treatment and subsequent visualisation of latent fingermarks. In addition, expanding the existing fundamental knowledge of the chemical composition of latent fingermarks may result in the development of more effective detection methods and enable more complex information to be derived from collected evidence.

Two amino acid sensitive reagent formulations were developed in the course of this PhD program. *p*-Dimethylaminobenzaldehyde (DMAB) was found to yield fingermark impressions on paper surfaces that are both coloured and photoluminescent. A wet contact method proved effective on non-fragile porous substrates such as white photocopy paper and various other substrates. Dry contact DMAB was shown to develop latent fingermark deposits on a range of substrates, but did not offer the same level of development as ninhydrin. A new and improved formulation of *p*-dimethylaminocinnamaldehyde (DMAC) was established for the detection of latent fingermarks. The low polarity of the solvent used, and heat-free reaction, enabled fingermark development on thermal paper without modification to the formulation. Comparisons to previously published formulations indicate that the new wet contact formulation affords a more rapid and sensitive development of latent fingermarks.

The amino acid variability in sweat, which may affect the ability of amino acid sensitive fingermark reagents to successfully develop all latent fingermarks within a large population, was investigated. Fingermarks which were treated with 1,2-indanedione/zinc chloride (IND/$ZnCl_2$) were ranked according to the developed sample's ridge detail and contrast using a 5-point grading study developed by the British Home Office. An initial pilot study examining samples from 120 donors reinforced that variation of amino acids in fingermark deposits exists within a population and is detectable through examination of IND/$ZnCl_2$ treatment and

subsequent Mann-Whitney U and Wilcoxon signed rank tests evaluation. Furthermore, Wilcoxon signed rank tests and intraclass correlation coefficients were used to show that the grading scale was an appropriate and consistent method to attain absolute values for developed latent fingermark samples. The donor study, consisting of 131 donors, showed that out of a total 1310 grades given to IND/$ZnCl_2$ treated fingermarks, only 0.5 % returned a score of 0. In agreement with the pilot study, fingermarks developed within 3 days were found to vary significantly between the age of the donor and the washing of hands prior to deposition. Donors who did not wash their hands the hour prior to deposition, or were below the age of 25, were more likely to offer higher grades.

The results of the pilot and donor studies highlighted the requirement for more specific chemical techniques to identify and profile the actual amino acid content rather than subjectively grading the reaction products. The detection of 21 amino acids using a high performance liquid chromatography with ultraviolet – diode array detector (HPLC-UV-DAD) was used in conjunction with liquid chromatography mass spectrometry (LC-MS). Fingermark samples from 50 donors were analysed, where it was found that serine was the most abundant amino acid in all samples, and that an average amino acid concentration of 520 ng per fingermark was detected.

Comparisons of the absolute and relative concentrations of the 5 most abundant amino acids (serine, glycine, ornithine, alanine, and aspartic acid), found that there were no significant differences due to food consumption, use of cosmetics, or biological sex with either LC method. HPLC-UV-DAD analysis suggested that the absolute concentration deposited by donors over and under the age of 25 was statistically dissimilar in these 5 amino acids, and also different for all relative amino acid concentrations (apart from alanine). No such dissimilarity was observed in the LC-MS results. Conversely, the absolute concentration deposited by donors who had and had not washed their hands within one hour of fingermark deposition were statistically dissimilar in 17 out of 20 amino acids according to LC-MS analysis, and in all analytes increased abundances were recorded from donors who had not washed their hands. This was not evident in the HPLC-UV-DAD results but was observed in the donor study.

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Publications

This thesis dissertation contains work which has been submitted for publication in peer reviewed journal articles.

P. Fritz, A.A. Frick, W. van Bronswijk, S.W. Lewis, A. Beaudoin, S. Bleay, and C. Lennard, *The variability and subjectivity of fingermark grading – towards a more consistent approach*. Journal Forensic Identification, 2014. Submitted.

P. Fritz, W. van Bronswijk, B. Dorakumbura, B. Hackshaw, and S.W. Lewis, *Evaluation of a solvent-free p-dimethylaminobenzaldehyde method for fingermark visualisation with a low cost light source suitable for remote locations*. Journal of Forensic Identification, 2015. 65(1): p. 67-90.

P. Fritz, W. van Bronswijk, D. Fisher, and S.W. Lewis, *Preliminary investigations into a commercial thermal fingerprint developer for the visualisation of latent fingermarks on paper substrates*. Journal of Forensic Identification, 2014. 64(6): p. 536-555.

P. Fritz, W. van Bronswijk and S.W. Lewis, *p-Dimethylaminobenzaldehyde: preliminary investigations into a novel reagent for the detection of latent fingermarks on paper surfaces*, Analytical Methods, 2013. 5(13): p. 3207-3215.

P. Fritz, W. van Bronswijk, E.L.T. Patton, and S.W. Lewis, *Variability in Visualization of Latent Fingermarks Developed with 1,2-Indanedione–Zinc Chloride*, Journal Forensic Identification, 2013. 63(6): p. 698-713.

A. Frick, **P. Fritz**, S.W. Lewis, and W. van Bronswijk, *Sequencing of a modified Oil red O Development technique for the detection of latent fingermarks on paper surfaces*, Journal Forensic Identification, 2013. 63(4): p.369-385.

S. Zadnik, W. van Bronswijk, A. Frick, **P. Fritz** and S.W. Lewis, *Fingermark simulants and their inherent problems: a comparison with latent fingermark deposits*, Journal of Forensic Identification, 2013. 63(5): p. 593-608.

P. Fritz, W. van Bronswjik, K. Lepkova, S. W. Lewis, K. F. Lim, D. E. Martin and L. Puskar, *Infrared Microscopy Studies of the Chemical Composition of Latent Fingermark Residues,* Microchemical Journal, 2012. 111: p. 40-46.

A. Frick, **P. Fritz**, and S.W. Lewis, *Chemistry of the Print Residue*, in Encyclopedia of Forensic Sciences, J.A. Siegel and P.J. Saukko, Editors. 2013. p. 92-97.

A. Frick, **P. Fritz**, S.W. Lewis, and W. van Bronswijk, *A modified Oil red O reagent for the detection of latent fingermarks on porous substrates*, Journal of Forensic Identification, 2012. 62(6): p. 623-641.

Conference Presentations

Selected aspects of the work contained within this thesis were also presented at the following conferences:

P. Fritz, W. van Bronswijk, F. Busetti, R. Herman and S. W. Lewis, *Profiling of amino acids in latent fingermark*, oral presentation at the 22nd Australian and New Zealand Forensic Science Society International Symposium on the Forensic Sciences, Adelaide, Australia, September 2014

P. Fritz, W. van Bronswijk and S. W. Lewis, *p-Dimethylaminobenzaldehyde: Preliminary investigations into a novel reagent for the detection of latent fingermarks on paper surfaces*, oral presentation at the 22nd Australian and New Zealand Forensic Science Society International Symposium on the Forensic Sciences, Adelaide, Australia, September 2014

P. Fritz, S. Zadnik, W. van Bronswijk, S. W. Lewis, K. F. Lim and D. E. Martin, *Synchrotron Infrared Microscopy Studies of the Chemical Composition of Latent Fingermark Residues: Temporal Changes and Fingermark Simulants*, oral presentation at the 21st Australian and New Zealand Forensic Science Society International Symposium on the Forensic Sciences, Hobart, Australia, September 2012

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Abbreviations

APCI	Atmospheric pressure chemical ionisation
CE	Collision energy
CE	Capillary electrophoresis
DAD	Diode array detector
DFO	1,8-Diazafluoren-9-one
DMAB	*p*-Dimethylaminobenzaldehyde
DMAC	*p*-Dimethylaminocinnamaldehyde
ESI	Electrospray ionisation
GC	Gas chromatography
HFE-7100	1-Methoxynonafluorobutane
HOPSDB	Home Office Police Scientific Development Branch
HPLC	High performance liquid chromatography
IFRG	International Fingerprint Research Group
IND/$ZnCl_2$	1,2-Indanedione/zinc chloride
IS	Internal standards
LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
MALDI-MS	Matrix assisted laser desorption/ionisation mass spectrometry
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NBD-chloride	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole
NIN	Ninhydrin
OPA	*o*-Phthaldialdehyde
ORO	Oil red O
PCA	Principal component analysis
PD	Physical developer
pI	Isoelectric point
PITC	Phenylisothiocyanate
SRM	Selected reaction monitoring
TEA	Triethylamine
TFD-2	Thermal Fingerprint Developer
UV	Ultraviolet

Chapter 1
Introduction

Portions of this Chapter have been published in the Encyclopaedia of Forensic Sciences and the Journal of Forensic Identification:

A. Frick, P. Fritz, and S.W. Lewis, *Chemistry of Print Residue*, in *Encyclopedia of Forensic Sciences*, J.A. Siegel, P.J. Saukko, and M.M. Houck, Editors. 2013, Academic Press: Waltham. p. 92-97.

S. Zadnik, W. van Bronswijk, A. Frick, P. Fritz and S.W. Lewis, *Fingermark simulants and their inherent problems: a comparison with latent fingermark deposits*, Journal of Forensic Identification, 2013. 63(5): p. 593-608.

Forensic science is an important aspect of the modern justice system and involves the application of science to legal issues [1]. For the successful application of forensic sciences to the dissemination of evidence, an event called the exchange principle has to occur. First stipulated by Edmund Locard, the transfer of trace evidence is summarised by:

> "No one can act with the force that the criminal act requires without leaving behind numerous signs of it…" [2].

This concept can be interpreted in that every physical contact that occurs between persons, objects and locales will result in the transfer of some material. This trace evidence includes blood, fibres, explosives residues and paint. However, one of the most commonly encountered form of usable evidence at volume crime scenes are latent (invisible) fingerprints or fingermarks [3].

The ridgelines seen in deposited fingermarks are formed by the friction ridge skin on the palmar surfaces of the hands [4]. These ridgelines are unique to individuals and are therefore extremely useful for identification purposes, as well as demonstrating that a contact has occurred [5]. As the most common form of fingermarks left at incident scenes are invisible to the naked eye, a range of physical and chemical treatment options have been developed to visualise the impressions [6]. The method of development depends on the nature of the fingermark deposited and the substrate on which it has been placed. Porous substrates, such as paper exhibits, are frequently encountered at incident scenes [6]. These surfaces are important forms of evidence as they are thought to tightly bind amino acids present in natural skin secretions to the cellulose. This provides a long lasting, robust medium and therefore constitutes the predominant amount of research for the development and subsequent visualisation of latent fingermarks [7].

As exchange often occurs with minute quantities of material transferred, there is a need for improved detection methods to capitalise on these exchanges. This can be achieved by both the improvement of existing and new fingermark detection methods, and by the advancement of our fundamental knowledge of this form of trace evidence. This advanced understanding may then be applied to systematically

develop fingermark detection methods and may make new approaches to the identification process possible.

This thesis outlines the research undertaken for the development of novel, and improvement of existing, amino acid sensitive fingermark reagents for porous surfaces. Furthermore, the application of analytical chemistry techniques to improve our current understanding of the amino acid content of latent fingermark deposits is described.

1.1 Fingermarks

Friction, or epidermal, ridges are an evolutionary advancement that allow us to better grip objects and increase the sensations felt by touch [8]. These friction ridges develop in the first 20 weeks of gestation and causes parts of the epidermis to be raised, which results in ridge detail to be observed in fingermarks (Figure 1.1). The epidermis is the outer skin layer [7, 8]. It is a physical barrier to the external environment and is both waterproof and resilient towards hostile attacks on the body, including bacterial and physical [8]. The inner dermis acts as a cushion to outside stresses, helps to regulate body temperature and prevents the skin from drying out by producing an oily substance called sebum [8]. The hypodermis is a third membrane layer often associated with skin, although it is not strictly part of it. The hypodermis anchors the dermis and epidermis to bone and muscle tissue and also provides blood flow and nerves to other skin layers [9].

Figure 1.1 Fingerprint with the characteristic fingerprint ridges [10]

In 1684, Nehemiah Grew, an English physician, published the first article on the presence of ridges in the skin of the palms and hands [4]. Only 100 years later did the German anatomist Johann Christoph Andreas Mayer discover that these characteristics were unique to every individual [4]. However, the first fingerprint to be used in a forensic context for the identification of a suspect was in Argentina in 1892, by chief police officer Juan Vucetich [11]. Other notable pioneers of the fingerprint sciences include Sir William James Herschel who is generally seen as the first individual to study the permanence of friction ridge skin and saw the potential for identification in the ridge detail. The field was advanced by others such as Dr Henry Faulds and Sir Francis Galton [4, 11]. Between them they studied the permanence, uniqueness, ability to be classified and their value as forensic evidence [11, 12]. Subsequently a fingerprint classification system was developed under the aegis of Sir Edward Henry [4] which formed the basis for the widespread use of fingerprints for identification. The identification is true even for identical twins, where DNA is not able to distinguish between individuals [4]. Because of these features, fingerprints have become invaluable to establish that a contact has occurred, and to link individuals to incident scenes.

The current methodology for the fingermark identification process is abbreviated as the ACE-V method, which refers to its four components: analysis, comparison, evaluation and verification [7, 13]. The mark is initially scrutinised for its quality and features (analysis), then compared to a known mark (comparison) and a decision is made as to their agreement (evaluation) [7]. The verification step refers to the quality assurances and quality controls that the examiner has to conform to in order practise as a fingerprint expert, such as proficiency tests, audits, etc., as the evaluation is a purely subjective matter [7]. The comparison of a known mark and an unknown mark is therefore based on the successive analysis of level 1, 2 and 3 detail (going from general patterns to intrinsic detail), whilst taking into account the fingermark quality, until the criteria of the country where the comparison is being made is met [12]. Level 1 detail refers to the overall pattern of the fingermark, level 2 mainly consists of minutiae (major ridge deviations such as ridge endings, etc.) and features such as scars [7]. Level 3 is comprised of features of the ridges, e.g. the alignment and shape of each ridge, and pore shape and positions. The acceptance criteria has

been a strongly debated issue, where early guidelines set by Locard essentially stipulated that 12 or more clear minutiae gives certainty to the identification for high quality fingermarks. With decreasing quality and features, the certainty is also reduced [7, 13]. Countries the world over, and a majority of European countries still follow this empirical rule, where the criterion points range from 7 to 16 features [7]. Following a 3 year study into the relevancy of a fixed number of criterion points in 1970 by the International Association for Identification, the following conclusion was drawn: "… no valid basis exists for requiring a predetermined minimum number of friction ridge characteristics that must be present in two impressions in order to establish positive identification." [7]. This has been augmented by omissions of the original empirical method, for example some types of minutiae are more selective than others, that absence of minutiae is of importance, certain qualitative features should be considered as well and that the general pattern produced as an effect of papillary lines produces unique patterns [7, 12]. Countries such as Australia, Canada and the U.S. have therefore adopted the holistic approach where the decision rests on the examiner as to whether a fingermark match can be made [7, 13].

Fingermarks are categorised into three types: latent, visible and plastic marks [14]. Visible or patent marks are visible to the naked eye and consist of substances such as paint and blood. Plastic marks are observed when a fingermark has been deposited on substances that retain or mould the friction ridge details, such as wax or soap. The most common form of fingermarks encountered at incident scenes are latent or invisible marks [1]. These need some form of chemical or physical treatment to make them more readily visible, resulting in the development of a variety of visualisation techniques. As these methods interact with the fingermark deposits, an understanding of their composition, and factors that may affect their composition, is vital for sensitive and reliable detection.

1.1.1 Sources of fingermark components

The deposited fingermark residue consists of both foreign contaminants and secretions of glands present in the skin. These secretions are the result of the eccrine, apocrine and sebaceous glands, of which only the eccrine gland is actually found on the palms and fingers (Figure 1.2) [15]. The sebaceous gland is present in skin found

throughout the body, especially the forehead and scalp, whereas the apocrine gland is mainly found in the axillary and perineal areas The secretions of these glands can be transferred onto the hands by touching these body parts prior to the deposition of the fingermark [16]. Due to the location of the glands, the apocrine secretions are rarely present in significant amounts in fingermark deposits and are therefore not an important target for fingermark detection.

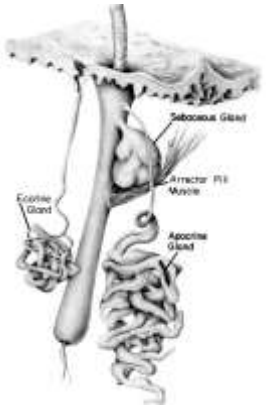


Figure 1.2 A schematic diagram of the three major secretory glands in relation to other cutaneous appendages. Reproduced with permission from Montagna and Parakkal [9].

The main roles of the eccrine glands are to cool the skin surface, excrete water, electrolytes and metabolites, and to protect against environmental hazards [9]. The sebaceous secretions inhibit the growth of bacteria, lubricate and protect the keratin in the hair shaft and conditions the surrounding skin [17]. The sebum also provides individuals with their unique scent signature and its production is primarily controlled by hormones, whereas the response of the eccrine glands can be stimulated by stress [9]. As shown in Table 1.1, an array of organic and inorganic constituents has been discovered to be secreted by these glands [15, 18-22].

Table 1.1 Constituents present in glandular secretions that may contribute to fingermark residues [15].

Gland type	Inorganic substituents	Organic substituents
Apocrine	Iron	Carbohydrates Cholesterol Proteins
Eccrine	Ammonia Chlorides Potassium Sodium Sulfates	Amino Acids Creatinine Lactic Acid Sugars Urea
Sebaceous		Alcohols Fatty Acids Glycerides Hydrocarbons

Sodium and potassium chloride are the most abundant compounds present in eccrine secretions and make up the majority of the inorganic salt content. Lactate is derived from glucose utilisation, and its presence indicates the activity of the eccrine gland. In addition, it is thought to be the target compound for cyanoacrylate fuming [23]. The presence of a range of amino acids in human sweat has been widely reported in the biomedical literature. Amino acids are of particular interest when considering the visualisation of latent fingermarks on paper, as they are the target compounds of routine detection methods [15]. Amino acids present in natural skin secretions are thought to bind tightly to the cellulose found in paper based substrates [7]. This provides a long lasting, robust impression and is therefore of special importance for the development and subsequent visualisation of latent fingermarks [7].

1.1.2 Deposited fingermark composition

In addition to the skin gland secretions, the actual composition of deposited fingermarks at the time of treatment depends on a variety of factors, including the type of substrate, deposition process, donor, ambient conditions, time and contaminants [24].

Substrates are broadly grouped according to the porosity of the surface, ranging from non-porous and semi-porous to porous. Non-porous substrates include glass and metal, whereas semi-porous materials include some plastics and waxed surfaces. Fingermarks deposited on non-porous substrates are typically treated by physical (e.g. dusting) rather than chemical means. Porous materials, such as paper and cardboard, are commonly treated with chemical reagents targeting the eccrine sweat [6]. Porous substrates not only contain fingermark residues on the surface, but eccrine sweat in particular is absorbed to some degree, offering long lasting impressions that may contain up to three times more amino acid material than non-porous substrates (Figure 1.3) [24]. When transferred to a porous substrate, the amino acids bind strongly without significant migration, which is key to affording reproducible ridge detail [25].

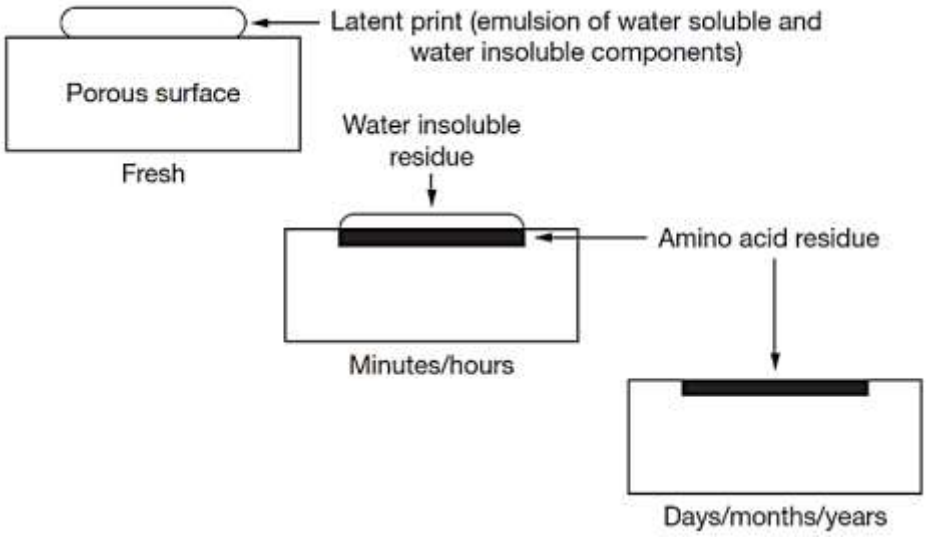


Figure 1.3 Schematic cross section of a latent fingermark on a paper substrate at various stages after deposition. Reproduced with permission from Lewis [26].

The act of depositing a fingermark on a surface is highly variable, resulting in additional deviations to the quality of the fingermark. Not only is the applied pressure of the fingertips onto the substrate important, but the contact angle, electrostatic forces, surface temperature and the duration of contact can influence the impression [3].

The individual depositing the fingermark also has a great effect on the amount and type of secretion which is deposited. Various studies suggest that there are significant compositional differences between individuals, in addition to changes over time within a single person [7, 24]. The amount of sebaceous material secreted in particular relies heavily on the age of the individual, as pre-pubescent children display relatively low sebaceous gland activity [27]. It has further been established that children deposit a higher proportion of volatile lipids, which evaporate more rapidly than the lipids deposited by adults [28]. In addition to the age of the donor, it is thought that the health, diet, medication and gender may play an important role on the chemical nature of fingermarks [7, 24].

With incidental contact of the fingertips with a vast array of chemicals occurring through everyday activities, these contaminants may also be present in latent fingermark deposits. In addition to chemicals arising from items such as food and cosmetics, chemicals of forensic value may also be present in the form of

contaminants [29, 30]. A range of studies have attempted and succeeded in the identification of recently related items such as explosive residues, fibres and drugs (or their metabolites) using a range of analytical instruments [20-22, 31].

After the deposition of a fingermark, environmental conditions play a large role in the longevity and quality of the impressions. Exposure to UV light, elevated temperature and humidity can affect the rate of degradation [7, 24]. In more extreme cases, such as the immersion of the fingermarks in water, or in cases of arson, successful identification from a fingermark can become much more difficult. As amino acids are soluble in water, lipid sensitive reagents are commonly used on substrates that have had contact with water [7, 15]. In the case of extreme heat, some studies have investigated the effect of pyrolysis on fingermark degradation [32]. While amino acids typically deteriorate at these temperatures, some of the pyrolytic products that can form may be targeted by fingermark reagents instead [32].

The time between fingermark deposition and analysis can result in the degradation of the deposit via evaporation of volatiles, oxidation, bacterial activity and the environmental factors discussed above [3, 30]. Volatile lipids and water evaporate relatively quickly, with up to 85 % of the fingermark evaporating within the first 2 weeks, resulting in a waxy, viscous residue [9]. Amino acids and salts present in eccrine sweat are non-volatile and crystallise on the surface, where UV light and airflow degrades them [33]. However, within one week, diffusion into porous substrates can prolong the stability of amino acids, with fingermarks on paper successfully developed after more than 20 years [6, 7]. In addition, some reagents react better with fresh fingermarks, while others develop increasingly well with an ageing mark [19]. The effect of ageing on fingermarks has not been extensively researched and a limited timeframe and small number of donors were used in those studies [18, 19].

Due to the presence and stability of amino acids on porous substrates, they have been a key target in studies involving fingermark deposits [30, 34-36]. These amino acids will be discussed in more detail below, due to their significant focus in this project and fingermark research in general.

1.2 Amino acids

The earliest documented observations of amino acids from hydrolysed proteins were in the early 19th century, and most of the standard amino acids were found within the next 100 years [37]. Amino acids were first discovered in human sweat in 1910 by Embden and Tachau, and by 1946 the majority of standard amino acids had been found by Hier, Cornbleet and Bergheim [38, 39]. Amino acids are essential to the functioning of the human body, as they are the building block precursors which allow the body to make proteins and peptides [40]. Additionally, amino acids perform other vital tasks in processes such as neurotransmitter transport and biosynthesis [41].

Amino acids are molecules which contain an amino and carboxylic acid group, as well as an organic side chain (Figure 1.4). The presence of these two functional groups in every amino acid allows these compounds the ability to take part in some important reactions, such as nucleophilic addition, imine and amide formation, and esterification [42, 43]. The specific side chains of each amino acid can undergo further reactions. Additionally, amino acids can react to form salts and take part in oxidation-reduction reactions, e.g. where cysteine can form disulfide bridges [42]. Chirality exists in all amino acids, apart from glycine, and these mirror images of the same amino acids are referred to as L- or D-amino acids. L-amino acids represent all amino acids found in proteins during translation in the ribosome, whereas D-amino acids are only found in some proteins and bacteria [42].

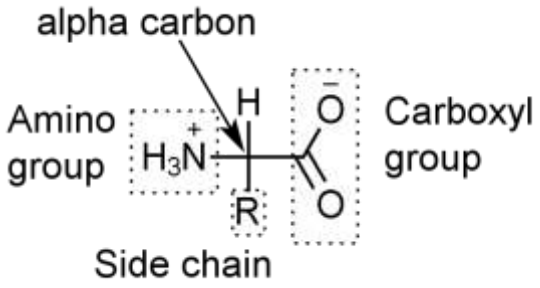
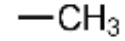
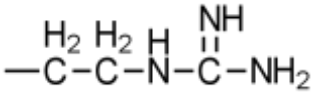
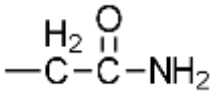
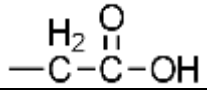
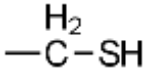
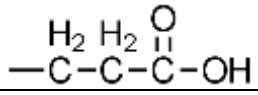
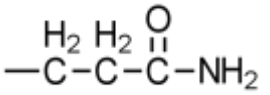
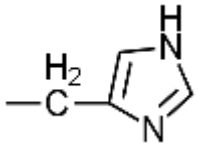
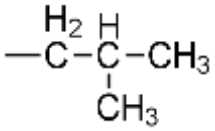
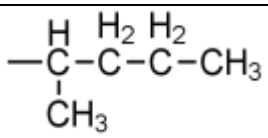
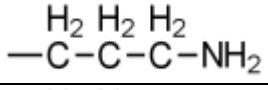
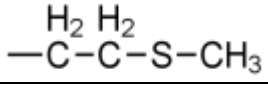
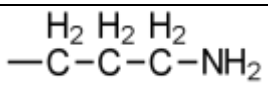
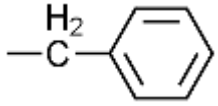
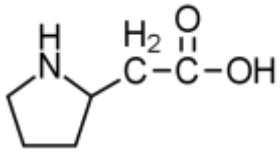


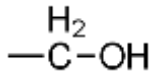
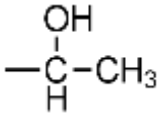
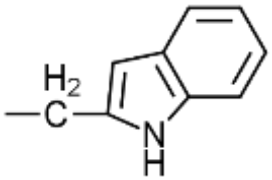
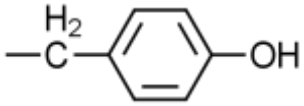
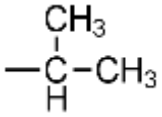
Figure 1.4 General structure of amino acids, where R is replaced by side chains to dictate the name and type of amino acid [41].

The neutral form of amino acids is very minor in aqueous solutions, with the zwitterionic form being dominant [42, 44]. At a pH range of 2 - 9, amino acids exist with the carboxylic acid being deprotonated to become a negative carboxylate ($-CO_2^-$) and the amino group being protonated to an ammonium group ($-NH_3^+$) for a net charge of 0 [42, 44]. At pHs lower than 2, the predominant form has a positive charge on the α-ammonium ion (and neutral carboxylic acid group), giving it an overall positive charge. At a pH of above 9, the opposite holds true where there is a negative charge on the carboxylate ion and a neutral ammonium group, giving rise to an overall negative charge [42, 44]. The isoelectric point (pI) is the pH at which an amino acid has no net charge; at a pH above the pI, the amino acid is negatively charged and at a pH below the pI it is positively charged [42].

Only 9 amino acids can be synthesised by the human body via common metabolic intermediates, these are called the non-essential or dispensable amino acids [45]. The others are essential or indispensable amino acids which need to be provided in the diet, apart from cysteine and tyrosine which are synthesised from indispensable amino acids. The amino acids listed in Table 1.2 are the 20 standard proteinogenic amino acids; however, others are used by the body [40, 45]. Several hundred other amino acid residues have been identified to occur in proteins, yet these are very limited and arise naturally from the encoded primary amino acids [46]. It is interesting to note that unlike the other standard amino acids, phenylalanine, tryptophan and tyrosine are naturally fluorescent [47]. Ornithine is a non-essential amino acid synthesised from glutamate, and is vital for the removal of excess nitrogen in the urea synthesis cycle [40, 48]. As it does not take part in protein building, it is known as a non-proteinogenic amino acid.

Table 1.2 List of 21 amino acids and their chemical and physical properties [41].

Amino Acid	Classification	Type	Structure of R group	Molecular Weight	pKa	pI
Alanine (Ala)	Non-essential	Aliphatic		89.09	3.60 10.19	6.0
Arginine (Arg)	Conditionally essential	Basic		174.20	2.18 9.09 13.2	10.8
Asparagine (Asn)	Conditionally essential	Amide		132.12	2.02 8.80	5.4
Aspartic acid (Asp)	Non-essential	Acidic		133.10	1.88 3.65 9.60	2.8
Cysteine (Cys)	Essential precursor	Sulfur containing		121.16	1.71 8.33 10.78	5.0
Glutamic acid (Glu)	Non-essential	Acidic		147.13	2.19 4.25 9.67	3.2
Glutamine (Gln)	Conditionally essential	Amide		146.15	2.17 9.13	5.7
Glycine (Gly)	Conditionally essential	Non-polar		75.07	2.34 9.60	6.0
Histidine (His)	Essential (for infants)	Basic		155.16	1.78 5.97 8.97	7.5
Isoleucine (Ile)	Essential	Aliphatic		131.17	2.32 9.76	6.0
Leucine (Leu)	Essential	Aliphatic		131.17	2.36 9.60	6.0
Lysine (Lys)	Essential	Basic		146.19	2.20 8.90 10.28	9.6
Methionine (Met)	Essential	Sulfur containing		149.21	2.28 9.21	5.7
Ornithine (Orn) [49]	Non-essential	Basic		132.16	1.94 8.65 10.76	9.7
Phenylalanine (Phe)	Essential	Aromatic		165.19	2.58 9.24	5.5
Proline (Pro)	Conditionally essential	Imino acid		115.13	1.99 10.60	6.3

Serine (Ser)	Conditionally essential	Hydroxyl		105.09	2.21 9.15	5.7
Threonine (Thr)	Essential	Hydroxyl		119.12	2.15 9.12	5.6
Tryptophan (Try)	Essential	Aromatic		204.22	2.38 9.39	5.9
Tyrosine (Tyr)	Conditionally essential	Aromatic		181.19	2.20 9.11 10.07	5.7
Valine (Val)	Essential	Aliphatic		117.15	2.29 9.72	6.0

1.2.1 Individual donor effect

To date, numerous medical studies have examined the effect of biological sex on the amino acid content in the human body, where a significant difference in some amino acids has been found in biological matrices, mainly blood [50-52]. For example, it has been noted that the levels of branched-chain amino acids are significantly lower in females than males. However, no gender related variation was identified in a recent work by Croxton *et al.* regarding amino acids in fingermark deposits [34].

Various studies have discussed the effect that the age of the donor has on the amino acid content in biological fluids [50-53]. In general, it has been noted that for females, a rise in the levels of amino acids in blood occurs with age, whereas the opposite holds true for males [50, 52]. In fingermark samples, Croxton *et al.* found that there were significant differences in the fingermark amino acid levels in the alanine, glycine and valine profile between donors 20 years and under and donors 21 years and over [34].

According to older studies, conducted in the 1940s for example, no appreciable connection between diet and amino acid excretion was observed, yet this may have been influenced by the instrument limitations of the time [39]. More recently, it has been shown that the amino acid content can vary with the diet of the donor [34, 54].

It has furthermore been established that the dietary intake of essential amino acids is critical to an individual's wellbeing, although this may not directly correlate to a difference in the content of amino acid excretions [41].

In addition to the diet, there are several illnesses which affect the production or presence of amino acids in the human body. Some of these diseases include β-thalassemia [55], colon carcinoma [56], Parkinsonism [57], the oxidative stress of amino acids [58] and haemoglobinopathy [59]. Amino acids can also be used as biomarkers in biological fluids as an indication of illness [53, 60].

Liappis and Hungerland observed that the amount of amino acids in sweat is affected by exercise and/or heat [61]. In their initial study of two adult males, they noted that the concentration of amino acids in sweat collected from the face was significantly higher after exercise, especially serine. Follow on studies by Liappis and Jaekel showed that this trend persisted across a larger, and more varied, donor pool [62]. It was furthermore observed that women had a larger increase in excretion of amino acids in sweat than males.

1.3 Amino acid analysis of latent fingermark deposits

A host of analytical chemistry methods for the detection and quantification of various analytes exist in the form of instrumental analysis [63]. These include gravimetry, potentiometry, atomic spectroscopy and spectrophotometry amongst others [63]. A further analytical method is chromatography, which is concerned with the separation of mixtures according to the difference in migration rates of the analytes through the stationary and mobile phase system [64]. To this effect, various techniques have been developed for industry and research purposes, of which gas chromatography – mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) are two of the most important approaches. In addition, matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and capillary electrophoresis (CE), although not chromatographic methods, have also seen use for the detection of amino acids in fingermark deposits in the past. Even though a myriad of other analytical techniques exist, these four approaches outlined above will be discussed in more detail below due to their use in the analysis of amino

acids in latent fingermark deposits. The concentration of amino acids in fingermark secretions has been reported to be between 0.3-2.6 ng/µL, or about 300 ng per fingermark, using a variety of methods [9]. This was found to be highly variable depending on the donor; in addition, the relative amounts of each amino acid can differ greatly.

1.3.1 Gas chromatography mass spectrometry

GC-MS has been widely used in the analytical community for the analysis of fingermark deposits [18, 27, 28, 32, 34, 65-72]. Amino acids found to date include alanine, aspartic acid, glycine, valine, leucine, isoleucine, serine, proline, asparagine, glutamic acid, phenylalanine, cysteine, p-chlorophenylalanine, lysine, ornithine, citrulline, methionine, histidine, arginine, tyrosine and threonine [32, 34, 68, 73, 74].

Croxton *et al.* in particular carried out comprehensive studies of amino acids and lipids present in fingermark deposits [34, 68]. In an initial study, various preparation methods for the simultaneous extraction of the lipid and amino acid fractions from substrates were compared. In 2010, Croxton *et al.* demonstrated that the sampling protocol for the fingermark collection is extremely important for the lipid constituents, as charged samples contained a 22-2000 % increase in total fatty acid content over uncharged deposits. However, there was minimal difference in the amino acid content of groomed or uncharged fingermarks, as the eccrine glands are predominantly present on the hands. They were less successful in analysing all the expected amino acids compared to the expected lipids due to the very low limits of detection required for the former. As such, only 11 out of the 20 standard amino acids were consistently found in all samples and gave reproducible values. Large inter-donor variability was also observed, with a range of 20.7 to 345.1 ng of total amino acid per fingermark identified in this study.

Richmond-Aylor *et al.* investigated the effect of pyrolysis on five of the most abundant amino acids (serine, lysine, alanine, glycine, and aspartic acid) present in latent fingermarks from a small number of donors [32]. As routine fingermark reagents are compromised when fingermarks have been subjected to very high temperatures (such as evidence from arson cases or on fired cartridge cases), the idea

was that novel fingermark reagents could possibly be developed to target these pyrolytic markers. These markers were identified in the pyrolysis products of alanine and aspartic acid in the form of 3,6-dimethylpiperazine-2,5-dione, maleimide and 2,5-furandione, respectively. Further studies are required to demonstrate the validity of these claims using a larger donor pool, more amino acids and a re-evaluation of the cotton glove substrate used for fingermark collection in this study.

Weyermann *et al.* used GC-MS to demonstrate that the choice of substrate has a dramatic effect on the quantity of sebaceous residue present for analysis. Non-porous surfaces displayed a decrease of up to 50 % in the total amount of residue deposited compared to porous substrates [72]. It was also noted that loss of material due to ageing occurred much more rapidly on non-porous surfaces. In addition to the substrate investigations, they noted a large variability of the initial sebaceous content between individuals. Whilst these observations were made with the sebaceous components of fingermark deposits, the substrate and intra-donor effects are likely to also exist for the amino acid content of these deposits.

1.3.2 Liquid chromatography

HPLC methods for the analysis of amino acids have existed since the 1950s, and have been improved and expanded on to accommodate the newer technology which has since been made available [75]. The concentration of free amino acids have been determined in various biological fluids, including blood, urine and sweat [76]. This work is of profound interest to a number of industries, including the food, pharmaceutical and biomedical disciplines.

To aid the analysis of amino acids for instruments equipped with ultraviolet or fluorescence detectors, derivatising agents are often used [76, 77]. While these reagents are also utilised for instruments using mass spectrometry detectors, due to their increased sensitivity and selectivity this may not always be necessary. A host of derivatisation agents exist, where two of the most commonly employed reagents are *o*-phthaldialdehyde (OPA) and phenylisothiocyanate (PITC) [77, 78]. OPA derivatisation is simple, sensitive, fast and reliable, but PITC is preferred when cysteine and secondary amino acid analysis is also required [79, 80].

A more recent paper by de Puit *et al.* reported the analysis of derivatised amino acids present in fingermark deposits from 20 donors using a liquid chromatography – mass spectrometry (LC-MS) instrument [35]. 19 natural amino acids were qualitatively and quantitatively evaluated, where arginine and glutamine were omitted. 9-fluorenylmethoxycarbonyl was used as the derivatising agent, where good separation was achieved (except for leucine/isoleucine) in 46 minutes. However, a method which assesses the amino acid (including arginine and glutamine) content of latent fingermarks, and applied to a larger donor population, is still required. As a range of recent studies have evaluated free amino acids in various biological fluids using LC-MS with underivatised samples, this may be of particular interest due to the simple sample preparation.

1.3.3 Capillary electrophoresis

The polar, zwitterionic amino acids are suited for capillary electrophoresis mass spectrometry (CE-MS) analysis, and a plethora of approaches have been published to this effect [81-87]. A large number of these studies focus on free amino acids in a variety of biological matrices, such as urine, tissue and sweat [85, 88, 89]. Traditionally, amino acids required derivatisation in order to achieve adequate retention, separation and sensitivity, and a variety of reagents are routinely used. However, in combination with mass spectrometry, complete baseline separation is not necessary in all cases to achieve good quantification and quantitation results. Common limits of detection (LOD) are in the micromolar range; however, some groups have reported nanomolar and even picomolar LOD for amino acid analysis following extensive sample preparation [82].

Atherton *et al.* have recently published a novel approach to the detection of amino acids in latent fingermarks by using CE-MS [36]. An optimised method for CE-MS was established to detect underivatised amino acids which were extracted from fingermarks deposited on Mylar® film. 12 amino acids were detected in fingermark samples, and of these 9 could be quantified. Samples were prepared as suggested by Croxton *et al.* for GC-MS analysis, except that the solvent changed to water and ethanol for CE compatibility [34, 36]. A lower LOD for the amino acid analysis of

latent fingermarks was achieved by GC-MS; however, the sample preparation was much more complex and arginine and histidine were not detected [34, 36].

1.3.4 Matrix assisted laser desorption/ionisation mass spectrometry

As MALDI-MS is a technique where the sample surface is ablated using a laser, the composition of the sample at the analyte/atmosphere interface is very important. In order to improve the sensitivity and resolution of the analyte, different matrix application techniques, such as microspotting or spraying the matrix either robotically or manually, have been developed for MALDI-MS [90-95]. Parameters that have to be controlled include deposition time, intervals, thickness of the matrix, wetness and drying time, as well as the chemical composition of the matrix [91]. Small, slowly formed crystals are preferable to increase signal intensity and resolution, but are dependent on the deposition method [91]. Furthermore, co-crystallisation between matrix and analyte, and analyte delocalisation, may also occur with some deposition methods [91].

The analysis of latent fingermarks using a two-step matrix deposition procedure has been outlined by Ferguson and co-workers [91]. The dry-wet method they used comprised of a powdered matrix being applied to the sample, which improves the total ion count by giving more homogenous matrix spots and smaller crystals [91, 92]. Subsequently, a solvent is sprayed on that dissolves both the analyte and matrix, providing efficient analyte extraction and co-crystallisation [91]. This method was deemed superior to previous spray-coating attempts, as fingermarks did not have to be previously enhanced or be on an appropriate MALDI substrate [91]. They conducted a validation study in 2013, linking particle size to MS quality and backing up their previous findings [96]. Ferguson *et al.* used MALDI to combine the identification process with chemical information, focussing on peptides and small proteins for the sex determination which they achieved with 85 % accuracy using multivariate modelling [97]. Wolstenholme *et al.* undertook a preliminary study into the effects of ageing on oleic acid at 4 °C, 37 °C and 60 °C for a seven day period [29]. The results indicated that at the two lower temperatures the signal intensity gradually declined, whereas at 60 °C there was rapid drop within the first day and then a relatively constant signal intensity [29]. A matrix-free method was devised by

Abel *et al.* [98]. This was used to analyse fingermarks that consisted primarily of eccrine sweat, as the donors wore nitrile gloves for several minutes prior to sample acquisition. However, there is no mention as to the size of their donor pool or a comparison between the analysis with or without the use of a matrix. As per the LC-MS methods, positive ion mode is detailed to analyse amino acids with greater sensitivity than the negative ion mode, as per Lim *et al.* [99].

1.4 Amino acid sensitive reagents

Fingermark development reagents play a vital role in the determination that a contact has occurred, and the subsequent identification of an individual based upon that contact. A multitude of different chemical treatments for the visualisation of fingermarks exist; however, only a select number of these are routinely used. Amino acid sensitive reagents are the primary form of developing fingermarks on porous substrates, due to the robust and long-lived nature of the amino acids binding with the cellulose [7].

For the operational applicability of new fingermark reagents, they should possess certain attributes to make them more amenable for the task. Ideally, they should be sensitive, give good initial and luminescent detail and contrast, have low toxicity and a safe working method, be quick and easy to apply and visualise, be applicable to a range of fingermark deposits/substrates/substrate conditions, be cost effective and non-destructive (both for the sample and possible subsequent analyses), possess good reagent stability, form stable fingermark impressions and be environmentally friendly [6, 7, 14, 100].

Although the range of existing methods address most of these issues [6, 101], current research is focusing on incorporating as many of the above attributes as possible into the next generation of fingermark reagents. They have been shown to be a sensitive, quick and efficient means of visualising latent fingermark deposits [6].

In an Australian context, two commonly used fingermark reagents are ninhydrin (NIN) and 1,2-indanedione (IND). These will be discussed in more detail below, in addition to some novel fingermark treatment options.

1.4.1 Ninhydrin

Siegfried Ruhemann first discovered NIN as an amino acid reagent in 1910, giving the coloured reaction product his name, "Ruhemann's purple" [102, 103]. It was not until the 1950s that work done by Oden and von Hofsten showed that NIN was useful as a fingermark development reagent [104]. The purple complex is formed by the reaction of NIN with the nitrogen of the amine group in amino acids, as seen in Figure 1.5 [105, 106]. As Ruhemann's purple is an active chelating agent that can form co-ordination complexes with metal ions, secondary metal salt treatment was performed [107, 108]. This resulted in a luminescent product that was easy to visualise with the right optical filters and gave good contrast and improved sensitivity and stability [105]. Although the relatively slow reaction rate of NIN with amino acids can be accelerated by the application of heat, an unwanted side reaction of NIN with some of the additives found in paper may also be increased [6]. As this side reaction is slower than the desired one, it is not found to cause excessive background staining as long as developed marks are recorded immediately [109]. Despite the improved development with metal post-treatment, a simpler and more robust method is most commonly used in routine police work. This formulation consists of approximately 0.5 % (w/v) NIN in a solvent (mostly alcohol, methylated spirits or HFE-7100), progressing to completion at 50-80 % relative humidity over 24-48 hours [15, 108]. NIN is commonly dissolved in a polar solvent; however, background staining on certain materials is much higher even with small amounts of polar solvents. To reduce the polarity of the working solution, non-polar co-solvents are commonly used to preserve the exhibits [103].

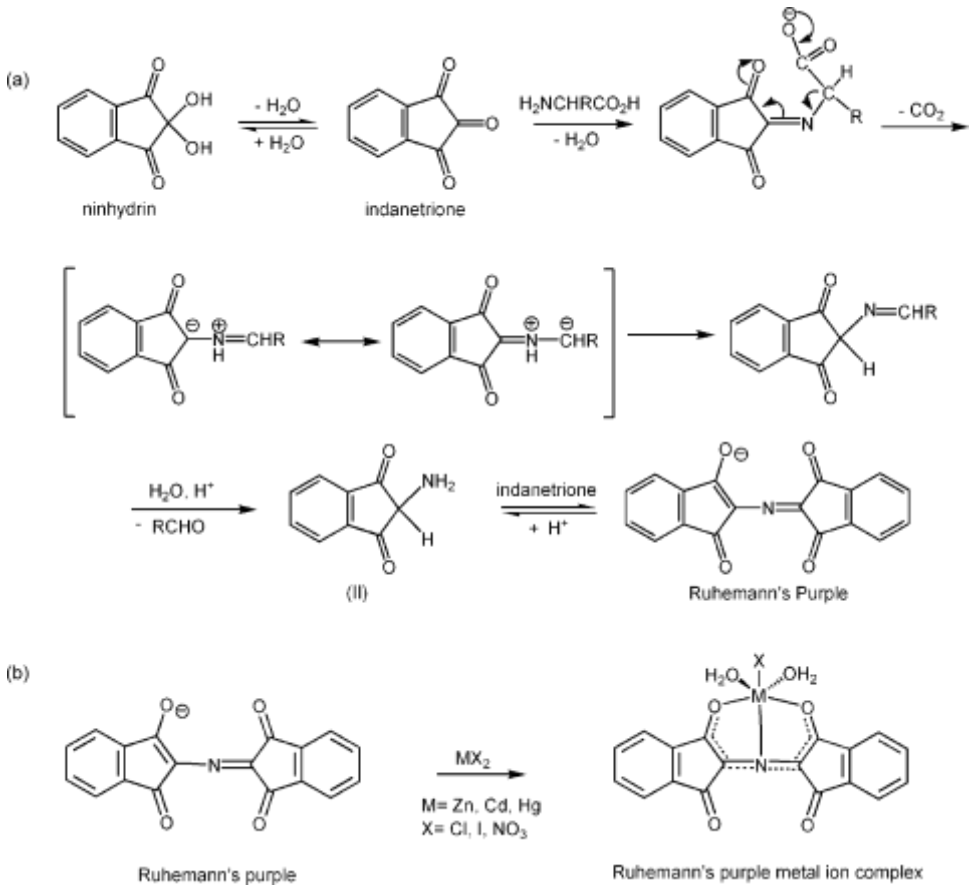


Figure 1.5 (a) The reaction mechanism of ninhydrin with amino acids to form Ruhemann's purple [103, 110, 111]. (b) The reaction of Ruhemann's purple with metal salts to form a complex ion [108, 112].

1.4.2 1,2-Indanedione

Fingermark chemists then turned their attention to finding ninhydrin analogues that were still capable of the Ruhemann's purple staining, but afford better contrast and sensitivity (Figure 1.6) [103, 105]. In 1997, Joullié *et al.* reported IND as a viable latent fingermark reagent [113]. While NIN provides superior colour under white light, IND is more sensitive and gives better contrast when viewing samples under luminescent conditions without further treatment [6]. Similar to NIN, the addition of a metal to the reagent improves the luminescence intensity and contrast, as well as improved longevity of the treated fingermark samples [107, 113, 114]. Unlike NIN, the metal salt is an integral part of the working solution, rather than additional post treatment step. Studies indicate zinc in the form of zinc chloride to be the metal of choice as it offers the greatest improvement in the luminescence intensity. Spindler *et al.* performed a comprehensive study where it was determined that zinc chloride acted as a Lewis acid catalyst [115].

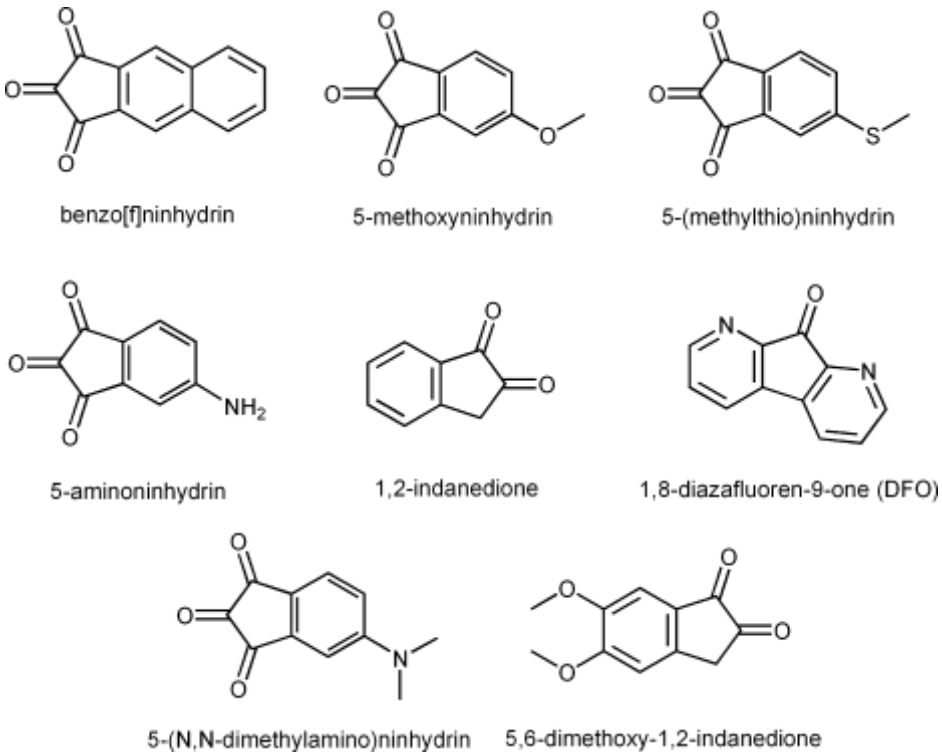


Figure 1.6 Structures of eight ninhydrin analogues [6].

Similar to NIN, an α–amino acid is converted into an aldehyde via an imine intermediate to give the pink reaction product, called Joullié Pink (Figure 1.7) [115]. However, unlike the Ruhemann's purple formation, the complex depends on the amino acid, where "R dependence" exists if one considers the general amino acid form of R-NH_2 [116, 117]. This general mechanism is referred to as a Stecker degradation.

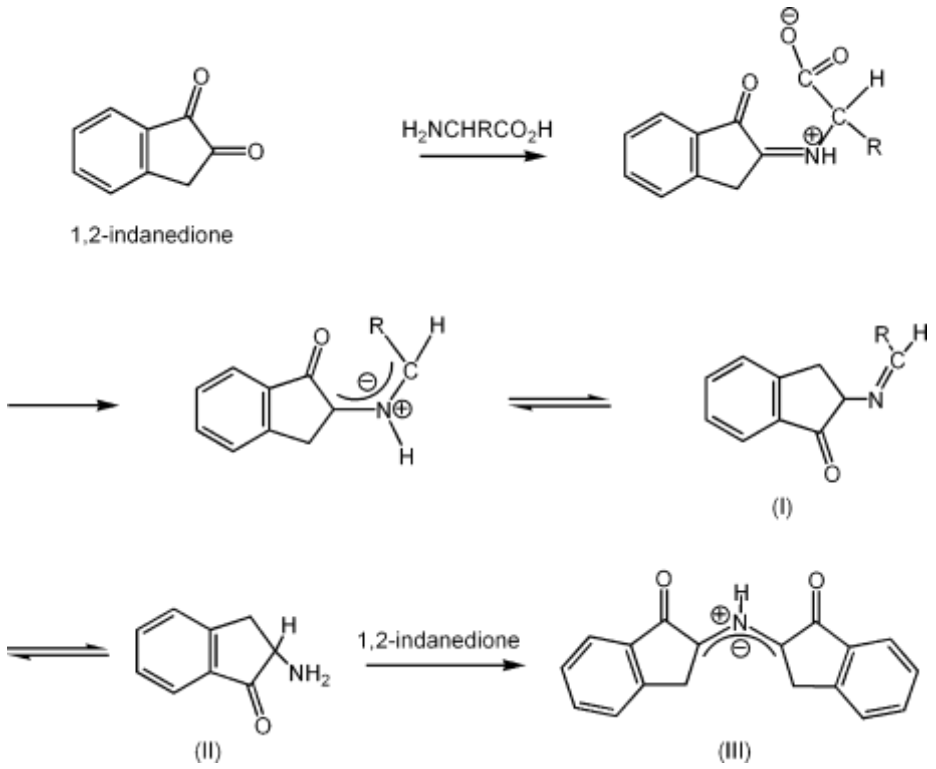


Figure 1.7 Proposed reaction mechanism of 1,2-indanedione and α-amino acids [117].

Although the 2' carbonyl site appears to be the preferential reaction site, recent research indicates that the difference in reactivity between the 1' and 2' carbonyl groups is minimal and therefore the reaction site is dictated by the strength of the nucleophile (Figure 1.8) [115, 118]. The strong nucleophile, together with an active and potentially catalytic substrate, is hypothesised to direct the formation of just the 2,2'- Joullié Pink isomer [115]. A cellulose matrix, such as paper, can further stabilise the reaction intermediates by acting as a surface catalyst, in addition to decreasing the degradation of the reaction product [115]. This is achieved by hydrogen bonding of the two carbonyl sites on the Joullié Pink ligands, which explains why these sites are already occupied and not available to form a metal co-ordination complex in a similar fashion to NIN [108, 115]. Furthermore, water molecules can be retained near the reaction site, while trace metals present from the paper production can also add to the catalysis of the reaction.

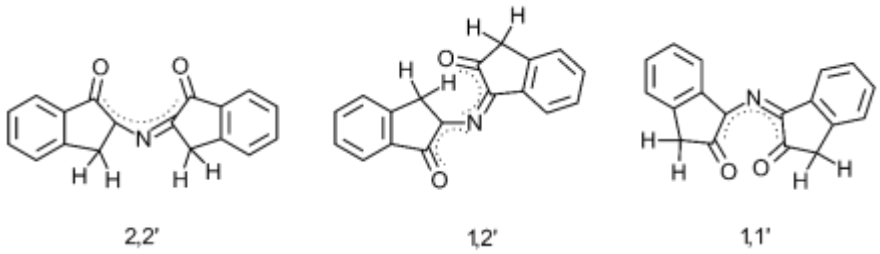


Figure 1.8 The three proposed structural isomers of Joullié Pink that could form from the reaction between 1,2-indanedione and α-amino acids. The enol tautomer of the 2,2'- isomer is hypothesised to be the major structure formed on eccrine-rich latent fingermarks [115].

The absorption (visible) band for the Joullié Pink complex is blue-green at 488 and 514.5 nm. Adding zinc chloride to the formulation causes a blue shift of the complex by 25 nm [119]. The maximum emission occurs between 560-570 nm, giving rise to a Stokes shift (difference between absorption and emission maxima) of around 120 nm. Laser-induced fluorescence results in photodegradation of the Joullié Pink complex emission peak at 564 nm, which was demonstrated by Alaoui using a IND-glycine solution in methanol, where the emission peak descended to a lower, stable level after about 1 hour of continuous excitation [120]. IND treated fingermarks were also irradiated for 5 minutes a day, where the emission maxima decreased in the first week and then remained relatively stable for nearly 2 weeks. Preliminary results indicate that there is no intermolecular energy transfer from the IND-glycine ligand to the zinc metal ion [120].

To further improve the development of latent fingermark deposits, changes of the reagent formulation have been undertaken since its initial proposal. These include alteration to the solvents used, the pH, as well as the application of heat and dry contact approaches. For the final working solution, two commonly used solvents are HFE-7100 and petroleum ether. The use of HFE-7100 can result in better development than HFC 4310mee, HFE 71de and methanol, and offers lower health and safety risks [107, 114]. Additionally, lipids are not dissolved by HFE-7100 which allows this formulation to be used in sequence with lipid sensitive reagents [107, 114]. Petroleum ether also offers very good contrast and fluorescence; however, the product is less stable than HFE-7100, and it is also highly flammable and toxic. Further solvents have been studied, yet these showed limitations in sensitivity through lower initial colour and/or luminescence, expense, toxicity, stability or sample preservation [107, 114].

In addition to the choice of solvent, the acidity of the formulation should be considered. As NIN requires a slightly acidic environment, this was also believed to be necessary for IND due to their structural similarity [6]. The acidified reagent is still widely used; however, Wiesner *et al.* showed that better results could be achieved without the use of acid [121].

Reaction time can be greatly reduced by applying heat to the treated samples. Development can be observed after 24-48 hours without heating, but only after 5 days will the results be similar to heat treated samples [114]. Heat can be applied using an oven or a laundry press, but the latter results in better luminescence and requires only 10 seconds at 160-165 °C compared to 10-20 minutes at 100 °C [107, 109, 114]. Samples developed at a higher humidity result in superior initial colour and fluorescence with both the HFE-7100 solvent and petroleum spirits [107, 114]. Studies have found that treated fingermarks are degraded by sunlight, with the intensity decreasing by 80 % after 28 days of exposure [122]. Similar to NIN, DNA can be successfully extracted from IND treated fingermarks within the first 5 days of treatment, where longer trial periods have not yet been successful [123].

Patton *et al.* presented a new technique for the application of IND called the dry contact method, which consists of sandwiching the samples between two treatment papers. The treatment papers are prepared by dipping plain white copy papers into an alternative IND reagent formulation and are subsequently leaving them to dry prior to use. This method has advantages in developing marks on thermal or very fragile paper and it can be easily transported if travelling is required [124]. Despite this, an acid-free wet contact formulation, which also does not use the application of heat, is the preferred method for thermal paper substrates, as recommended by the National Centre for Forensic Studies [109].

1.4.3 Alternative fingermark reagents

Although ninhydrin, 1,2-indanedione, and to a lesser extent 1,8-diazafluoren-9-one, have found practical application in the visualisation of fingermark deposits, a range of alternative fingermark reagents have been proposed over the last 50 years to overcome some of the limitations discussed above [101, 125, 126]. Some of the more promising alternatives to ninhydrin and its analogues include amino acid assays such as fluorescamine, o-phthalaldehyde, NBD-chloride (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) and *p*-dimethylaminocinnamaldehyde (DMAC), as well as reagents based on natural compounds such as genipin and lawsone (Figure 1.9) [6].

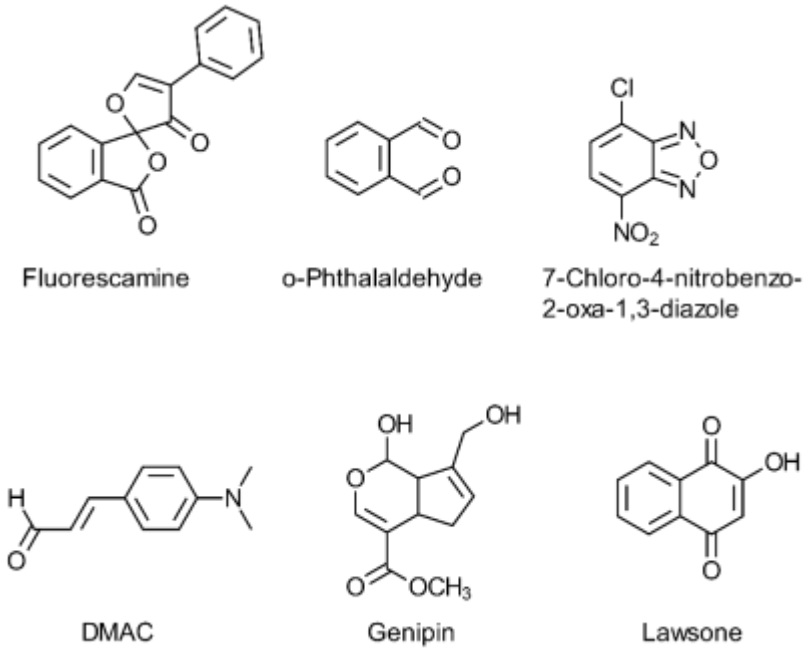


Figure 1.9 Structures of select alternative amino acid sensitive fingermark reagents [125, 127].

Extracted from the fruit of *Gardenia jasminoides*, genipin was investigated in several publications over the last decade to discern its use as a novel fingermark reagent [128-130]. Initially studied by Almog *et al.*, it showed promise due to its luminescence and safety benefits over established methods [128]. The clear working solution was shown to provide strong luminescence at an emission wavelength of 620 nm when excited at 590 nm. This research was followed on with changes to the formulation and more in-depth studies on its applicability on a range of substrates. In effect, it was found that genipin was not as sensitive as existing methods; however, it may offer advantages with substrates which have strong self-fluorescence and benefit

from visualisation at longer wavelengths [129]. This work did prompt the investigation of other, natural compounds for the visualisation of latent fingermarks, for example lawsone [127].

Lawsone (2-hydroxy-1,4-naphthoquinone) is the active compound within henna, a natural skin and hair dye [127]. Based on a class of compounds called napthoquinines, which are known for their reactions with amino acids, lawsone was hoped to offer a novel method for the detection of fingermark deposits on porous substrates [127]. A formulation of 1 g L^{-1} lawsone in 20:80 % ethyl acetate: HFE-7100 was found to give the most development in the preliminary study, where emission occurred at 640 nm after excitation 590 nm, similar to genipin. Despite this initial promise, lawsone was found to be less sensitive than existing methods and was not further pursued [127].

Fluorescamine was first investigated in the context of developing fingermarks in 1979 by Lee and Attard, and later re-evaluated by the Home Office Centre for Applied Science and Technology [125]. In 1972, Weigele reported the use of fluorescamine for the detection of primary amino acids [131]. Based upon this information, Lee and Attard proposed a novel fingermark reagent as a ninhydrin alternative [126]. Although the resultant marks were found to be more strongly developed on certain substrates than ninhydrin, the samples were less stable. In addition, the use of UV light sources for the excitation was a further disadvantage and this method ultimately was not recommended for routine police work [125].

o-Phthalaldehyde was studied by Mayer *et al.* as an alternative amino acid sensitive fingermark reagent in 1978 [132]. This work was based on the amino acid assay developed by Benson and Hare in 1975, which was found to be more sensitive, stable and cheaper than fluorescamine [133]. In the presence of 2-mercaptoethanol, it reacts with primary amino acids to yield luminescence at 455 nm when excited with UV radiation at 340 nm [132]. It is postulated that the increased use of optical brighteners in paper substrates, which can be brighter than the luminescence of treated fingermark deposits, are partly to blame for this method's lack of use by the forensic community [125].

A further amino acid assay, NBD-chloride, was investigated as a viable option for fingermark treatment by Salares *et al.* [134]. Similar to the development of fluorescamine and o-phthalaldehyde, this work was largely based on the results of amino acid detection in the biochemistry field [135]. Further work by the Forensic Science Research Unit in Canberra, Australia, in the 1980s improved upon the original method by using a cheaper excitation source and compared it against ninhydrin on a range of samples [136, 137]. Despite similar development being observed, and improved contrast on older deposits, its significant health and safety risks (potent mutagen) ultimately resulted in the rejection of this method [136, 138].

DMAC was proposed by Morris and Goode in 1973 as a fingermark reagent via its reaction with the deposits to form an imine [139]. Initial tests did not show any improvements over the performance of ninhydrin, yet deposits older than 72 hours could not be reliably developed [23]. A range of different formulations have been proposed since then, where the sensitivity and visualisation of older impressions have been improved [23, 140-143]. Most of the approaches have focussed on the dry contact methods, which make it amenable to thermal paper treatment. The lack of recent wet contact approaches and its inherent properties of DMAC prompted a new investigation into its formulation and method of application in Chapter 3.

1.5 Fingermark simulants

As has been discussed in section 1.3, latent fingermark deposits are extremely variable in nature. It is therefore difficult to obtain samples of consistent composition and quality for the purposes of reagent and formulation comparisons, and for quality assurance purposes [144]. Fingermark simulants have been proposed for use in the method development and validation stages of amino acid sensitive reagents, with the view of being unchanging artificial standards [145-148].

A variety of these fingermark simulants have thus been developed, in order to mimic the eccrine secretions present in fingermark deposits [145-148]. For example, Olsen initially proposed to use beef broth to test the response of ninhydrin [149]. More advanced methods consist of either single or multiple amino acid mixtures of different concentrations, which are subsequently spotted onto a substrate [147, 148,

150]. In some commercial simulants, it has been found that the total concentration of the amino acids is much higher than in latent fingermark deposits, where they only comprise about 1 % of the total mixture [151]. In addition, fingermarks consist of a complex mixture of eccrine and sebaceous secretions, rather than just individual amino acids, which further affects the ability of reagents to visualise the deposits. As the work by Zadnik *et al.* indicates, simulants do not offer realistic imitations of fingermark deposits when treated with some fingermark reagents [151]. Although 1,2-indanedione treated samples develop with the expected colour, the product is much more intense, suggesting a higher concentration of the amino acids. In the case of ninhydrin, the orange colour of the treated simulant indicates that the reaction product is significantly different to treated latent fingermark deposits which generally display purple ridge detail. It should be noted that spot tests in addition to latent fingermark samples are useful in order to give an indication of reagent efficacy; however, studies into developing improved and more realistic simulants are required before they can be confidently used as independent fingermark substitutes.

1.6 Aims

Amino acids are the primary targets of chemical visualisation methods for fingermarks deposited on porous substrates. As such, the impetus of this research was to develop novel reagents and to further investigate the amino acid content of fingermark deposits towards improving detection methods.

Chapters 2 and 3 describe the development of a novel fingermark reagent (*p*-dimethylaminobenzaldehyde) and substantial improvements to the formulation of *p*-dimethylaminocinnamaldehyde. Both amino acid sensitive reagents will be evaluated for their performance on fingermarks deposited on a range of substrates and in comparison to existing treatment options. One of the existing amino acid sensitive reagents, 1,2-indanedione/zinc chloride, will be used to show the amino acid variation of treated fingermarks that exists within a large population of donors in Chapter 4. The results focus on the evaluation of the data to establish possible correlations between fingermark development and donor traits and habits.

Chapters 5 and 6 will show the development and application of analytical techniques, in particular liquid chromatography mass spectrometry (LC-MS) and high performance liquid chromatography coupled with an ultraviolet - diode array detector (HPLC-UV-DAD), to qualitatively and quantitatively examine the profile of 21 amino acids in a population of fingermark donors. The data will be evaluated to correlate the traits and habits of the donor as a function of the amino acid content of the deposits.

Chapter 2
p-Dimethylaminobenzaldehyde: evaluation of a novel luminescent fingermark reagent

Portions of this Chapter have been published in the following journals:

P. Fritz, W. van Bronswijk, B. Dorakumbura, B. Hackshaw, and S.W. Lewis, *Evaluation of a solvent-free p-dimethylaminobenzaldehyde method for fingermark visualisation with a low cost light source suitable for remote locations.* Journal of Forensic Identification, 2015. **65**(1): p. 67-90.

P. Fritz, W. van Bronswijk, and S.W. Lewis, *p-Dimethylaminobenzaldehyde: preliminary investigations into a novel reagent for the detection of latent fingermarks on paper surfaces.* Analytical Methods, 2012. **5**(13): p. 3207-3215.

2.1 Introduction

The visualisation of latent fingermarks on surfaces by chemical means can be considered to be the trace detection of the various biomolecules and excretions from the skin secretions. These make up the latent impressions where their spatial distribution needs to be retained for subsequent analysis [6]. Fingermark detection chemistry shares with other areas of analytical chemistry the constant search for improved selectivity and sensitivity in order to maximise the number and quality of latent fingermarks detected on exhibits [127, 152-154]. The substrate upon which a latent fingermark is deposited has a significant influence on the nature of the detection technique applied [24, 155]. Detection methods that target amino acids present in latent fingermark deposits have achieved widespread use for paper surfaces due to the strong and stable binding of free amino acids onto cellulose fibres resulting in a good representation of the fingermark [6, 103, 113, 156]. As alluded to in Chapter 1, existing visualisation methods can still benefit from improvements regarding safety, sensitivity, cost and simplicity by changes in their formulations or by pursuing novel fingermark treatment options. One of these potential alternative reagents is *p*-dimethylaminobenzaldehyde (DMAB) (Figure 2.1).

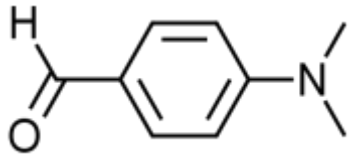


Figure 2.1 Chemical structure of DMAB.

2.1.1 *p*-Dimethylaminobenzaldehyde

A recent article by Takatsu *et al.* discussing the fuming of latent fingermarks with cyanoacrylate ester and the subsequent aid in visualisation using DMAB inspired the pursuit for alternative reagents based on the dimethylamino group [157]. In that particular study, it was thought that DMAB reacted selectively with cyanoacrylate ester fibres on fingermark ridges, as fumed blank (no fingermark deposit) samples gave no positive result. Takatsu *et al.* argued that the fibrous form of the superglue was responsible for the greater amount of adhesion of DMAB to the substrate, which would imply that no chemical reaction was taking place and that the DMAB molecule itself is luminescent at an excitation wavelength of 365 nm [157].

DMAB is primarily utilised in histochemical studies to visualise amines in-situ through both colour and photoluminescence [158-162]. Due to DMAB's use in a range of stains such as Ehrlich's Reagent, where DMAB reacts with primary and secondary amines, it is postulated that rather than a process of simple adhesion to the cyanoacrylate fibres, it may actually react with the amino acid content of latent fingermark deposits. This suggests the potential to use this compound as the basis for a development technique for latent fingermarks, both through its ability to produce photoluminescent products with the target analytes as well as the maintenance of spatial integrity, which is essential for fingermark identification. As DMAB is also used extensively on micro-organisms such as bacteria, it is unlikely to be spatially hindered by the micrometre sized deposits which fingermark ridges consist of [162].

2.1.2 Reaction pathway

The DMAB reaction with primary amine groups has been widely studied in the biological field [158-161]. This reaction, called an imine or Schiff base formation, occurs in acidic conditions where the slightly basic DMAB molecule is protonated by a weak acid to initiate the reaction [163]. It is generally accepted that in biological samples a working solution pH of 4-5 provides the best reaction [43, 164]. If the conditions are too acidic, the nucleophile (i.e. amino acids) are fully protonated and therefore unreactive. If the conditions are too basic, the proton catalysed dehydration of DMAB is impeded [165]. DMAB bonds to the nitrogen present in amine groups such as amino acids and urea, and the general reaction mechanism is shown in Figure 2.2. It can also react with secondary amines to produce enamines, where a proton is lost from the adjacent carbon instead of the nitrogen [43]. DMAB reacts to give a yellow product with monoamines and an orange one with polyamines [163].

The colour is caused by an intramolecular charge transfer, which can facilitate the excitation of an electron. This is true for increasingly conjugated molecules, where a 'push-pull' system can occur. In this case, the colour of these compounds originates from π- π^* electronic transitions, which likely possess a significant charge transfer character due to the presence of the electron donating dimethylamine and the electron withdrawing imine nitrogen. The strength of these electron donating and

withdrawing groups, as well as the length of the conjugated chain between these groups, affect the colour and intensity of the product.

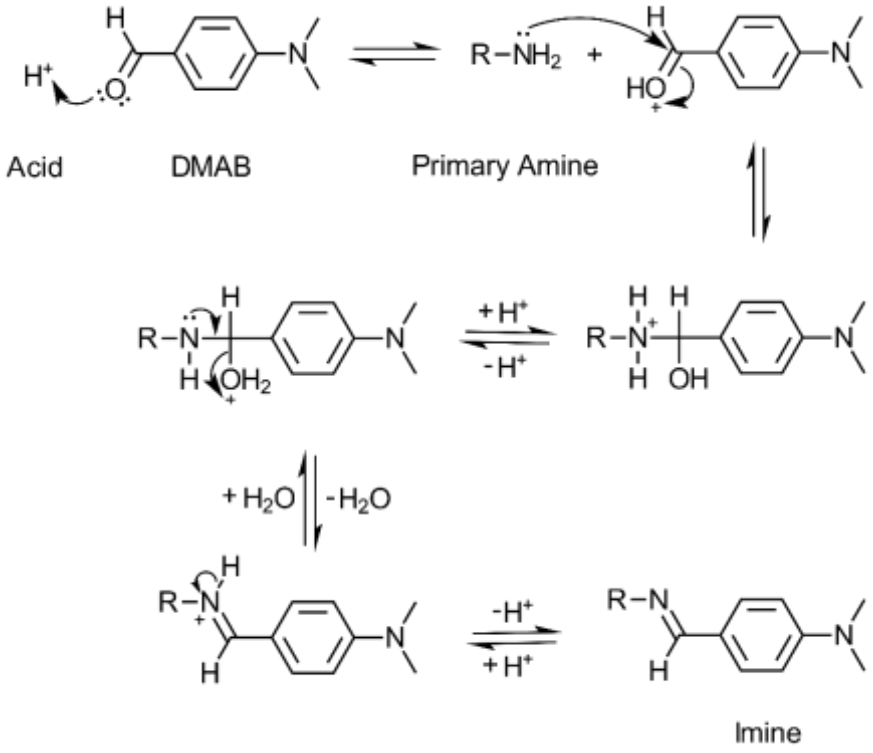


Figure 2.2 General reaction mechanism for the imine formation from primary amines and DMAB (adapted from Adegoke and Nwoke, 2008) [166].

2.1.3 Experimental design

To aid the experimental design of these studies, the recent special feature report published by the International Fingerprint Research Group (IFRG) was considered [100]. In that report, guidelines are proposed by the IFRG to the best practice approach for the evaluation of novel or modified fingermark treatment options. To summarise, the evaluation process can be divided into four phases. Phase 1, or pilot studies, can be considered proof-of-concept investigations and do not require rigorous testing for this initial work. However, before the potential of a novel technique is subjected to a full validation study under pseudo-operational conditions (Phase 3), it is evaluated using a larger number of donors, substrates and other variables to satisfy the criteria of a Phase 2 study. Lastly, Phase 4 assessments are implemented as the final step and involve casework trials and the "inclusion into standard operating procedures" [100]. The document therefore provides a list of

guidelines, which facilitate maintaining and improving the quality of studies being conducted and presented by the fingermark research community. The continuous quest for alternative and improved methods is the primary focus of fingermark research for the practical advancement of operational casework facilities [167].

2.1.4 Alternative light source

Anecdotally, one limitation to the widespread use of luminescent fingermark reagents is the expensive nature of forensic light sources, especially in low volume forensic laboratories. It has been well established that the increased contrast and decreased background interference of luminescent fingermarks are very useful for routine police work, especially with deposits on brightly coloured or patterned substrates [6, 14, 129]. Recent, unpublished experiments by the author have identified a cheap, commercially available, LED light source that is capable of causing luminescence in 1,2-indanedione-zinc chloride (IND/$ZnCl_2$) treated exhibits. Due to the low cost (approximately US$ 25), robustness and portable nature of this light source (available in a range of output colours depending on the excitation wavelength required), it presents a very appealing case for those laboratories where more expensive and cumbersome systems are not feasible.

2.1.5 Aims

This Chapter presents preliminary studies into the application of DMAB as a reagent for the detection of latent fingermarks on paper surfaces. Dry and wet contact approaches to using DMAB will be discussed in detail. The study presented here also provides a demonstration of how the IFRG guidelines can be used to assist planning and implementation of an evaluation of a potential fingermark visualisation treatment. All DMAB dry contact images were also recorded using the alternative LED illumination to assess its practical use for fingermark casework.

2.2 Materials and methods

2.2.1 Chemicals

1,2-Indanedione (CASALI/Optimum Technology, Australia), absolute ethanol (CSR chemicals, Australia), acetone (Ajax Finechem, Australia), l-alanine (BDH, Australia), l-serine (Sigma-Aldrich, USA), anhydrous zinc chloride (BDH, USA),

citric acid (Ajax Finechem, Australia), ethyl acetate (Univar analytical, Australia), ferric nitrate nonahydrate (Chem-Supply, Australia), ferrous ammonium sulphate hexahydrate (Sigma-Aldrich, USA), glacial acetic acid (CSR chemicals, Australia), glycine (BDH, Australia), HFE-7100™ (1-methoxynonafluorobutane, 3M Novec, Australia), hydrochloric acid (Ajax Finechem, Australia), maleic acid (Sigma-Aldrich, USA), methanol (Mallinckrodt, USA), n-dodecylamine acetate (Optimum Technology, Australia), ninhydrin (Optimum Technology, Australia), Oil red O (Sigma-Aldrich, USA), *p*-dimethylaminobenzaldehyde (BDH, USA), petroleum spirits 40-60 °C and 60–80 °C (APS chemicals, Australia), propylene glycol (Sigma-Aldrich, USA), silver nitrate (Chem-Supply, Australia), sulfuric acid (Ajax Finechem, Australia) and Tween 20 (Sigma-Aldrich, Australia) were all used as received and were of analytical reagent grade unless otherwise stated.

2.2.2 Substrates

The substrates used in this study consisted of white copy paper (Fuji Xerox Professional, 80 g/m^2), newspaper, glossy paper (brochure), gift wrapping paper (various designs), Post-it® notes (various colours) and envelopes (various colours). Thermal paper was used in the form of unprinted thermal register rolls (Officeworks, Australia) and printed receipts from a number of supermarkets.

2.2.3 Collection of latent fingermarks

All fingermark donors were required to sign a Human Resources Ethics Committee approval form prior to the collection of any samples (Approval Number SMEC-94-11). Donors were asked to rub their hands together before deposition in an attempt to provide a more uniform distribution of the eccrine secretions. These deposits are referred to as natural or uncharged fingermarks, whereas those loaded with sebaceous secretions (i.e. by rubbing the hands on their face or hair) are called charged fingermarks in this thesis. Donors had not washed their hands, consumed food or handled chemicals in the 30 minutes prior to providing samples. Donors were instructed to gently place fingertips onto the substrate, and to not remove their hands until fingers had been outlined with a graphite pencil. Samples were treated within 24-36 hours following deposition, unless stated otherwise.

Latent fingermarks were collected on white copy paper (Fuji Xerox Professional, 80 g/m^2) from 2-10 donors per experiment for the wet contact DMAB method. Split prints were used for these preliminary studies as recommended by the IFRG [100] (Figure 2.3 a).

Latent fingermarks were collected on all substrates from at least 10 donors per experiment for the DMAB dry contact validation study. For the study of aged specimens, fingermarks were collected on plain white copy paper and kept in a darkened cupboard under controlled laboratory conditions for 6 and 18 months. For the blind study, donors were asked to deposit between 5-10 fingermarks on A5 sized white copy paper with each hand. The total number of marks that were deposited by each hand was noted by the donor, so that the researcher evaluating the blind study was not aware of the exact number of deposits. Three prints were collected from each hand for the depletion series, where 5 consecutive deposits were collected. One half of each hand was subsequently developed by DMAB and compared to the ninhydrin treated half. For this validation work, the 3 print approach was taken, where donors deposit index, middle and ring fingers onto the substrate, with the sample being cut along the middle finger (Figure 2.3 b) [100].

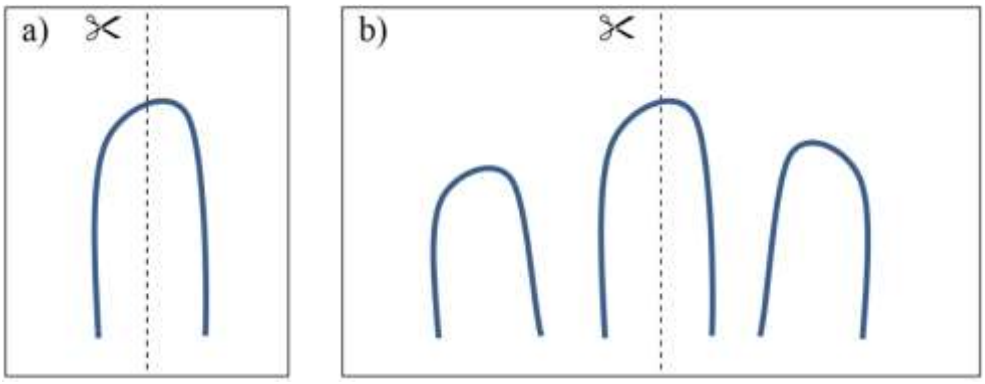


Figure 2.3 Outline of the finger placement on a substrate for sample collection of the split-print approach (a) and the 3-print approach (b).

2.2.4 Preparation of reagent solutions

The preparation of stock and working solutions for the DMAB formulations are summarised in Table 2.1. To prepare the treatment papers for the dry contact DMAB method, white copy paper was dipped into the working solution and allowed to air dry before being stored in a sealed zip-lock plastic bag.

Wet contact IND/$ZnCl_2$ reagent was prepared as recommended by the National Centre for Forensic Studies [6, 109, 124, 168]. Dry contact 1,2-indanedione (IND) reagent was prepared as described by Patton *et al.* with two formulations, one containing HFE-7100 and another containing petroleum spirits, being used [124]. White copy paper was dipped in the working solution, air dried and stored in a sealed zip-lock plastic bag to produce the treatment papers.

The formulation and application of ninhydrin (NIN) utilised for this project was as per the HFE formula prescribed by the National Centre for Forensic Studies [109]. Dry contact ninhydrin treatment papers, for use on thermal paper, were created by dipping white copy paper into the working solution and allowing the sheets to air dry before storing in a sealed zip-lock plastic bag.

The Oil red O (ORO) reagent was prepared as described by Frick *et al.* [169]. The stain solution was stored at room temperature in Schott bottles wrapped in aluminium foil.

Physical developer (PD) stock and working solutions were prepared as recommended by the National Centre for Forensic Studies [109], with a modification as described by Sauzier *et al.*, where Tween 20 was substituted for Synperonic N. [170]. The PD working solution was prepared fresh as needed, and only used twice before discarding.

Table 2.1 Preparation of stock and working solutions

	Solution	Reagent Preparation
Wet Contact DMAB	*DMAB stock solution*	1 g DMAB in 22 mL ethyl acetate and 3 mL acetic acid
	Working solution	1 mL stock solution diluted with 9 mL HFE-7100
Dry Contact DMAB	*Working solution*	2 g DMAB in 100 mL ethyl acetate
Wet Contact IND/$ZnCl_2$	*IND stock solution*	4 g 1,2-indanedione dissolved in 450 mL ethyl acetate and 50 mL glacial acetic acid
	$ZnCl_2$ stock solution	8 g zinc chloride dissolved in 200 mL absolute ethanol
	Working solution	2 mL zinc chloride stock solution and 50 mL stock solution added to 450 mL HFE-7100 solvent
Dry Contact IND/$ZnCl_2$	*1,2-indanedione stock solution*	0.75 g 1,2-indanedione and 20 mg $ZnCl_2$ dissolved in 0.5 mL ethanol, 15 mL dichloromethane and 35 mL ethyl acetate
	Working solution	5 mL stock solution added to 45 mL HFE-7100 or 45 mL petroleum spirits
Ninhydrin	*Ninhydrin stock solution*	36.5 g ninhydrin dissolved in 425 mL absolute ethanol, addition of 35 mL ethyl acetate followed by 40 mL acetic acid
	Working solution	65 mL stock solution is added to 935 mL HFE-7100
Oil red O	*Working solution*	0.05 g ORO dissolved in 100 mL propylene glycol at 95 °C with constant stirring. Cooled solution is vacuum filtered before use
Physical Developer	*Detergent-surfactant solution*	0.5 g n-dodecylamine acetate and 0.5 g Tween 20 dissolved in 125 mL deionised water
	Redox solution	7.5 g ferric nitrate nonahydrate, 20 g ferrous ammonium sulphate hexahydrate, 5 g citric acid and 10 mL detergent-surfactant solution dissolved in 225 mL deionised water in order given
	Silver nitrate solution	10 g silver nitrate dissolved in 50 mL deionised water
	Maleic acid pre-wash	6.25 g maleic acid dissolved in 250 L deionised water
	Working solution	7.5 mL silver nitrate stock solution added to 142.5 mL redox stock solution

2.2.5 Development of latent fingermarks using the IND methods

Samples developed with the conventional wet contact IND/$ZnCl_2$ method were dipped briefly in the working solution, and allowed to air dry before being heat treated for 10 seconds with an Elna laundry press (set at 160 °C) [109].

Dry contact IND treatment was carried out as described by Patton *et al.* [124]. Samples were sandwiched between two treatment papers and stored in a zip-lock plastic bag for at least 24-36 hours in the dark, with no heat being applied.

2.2.6 Development of latent fingermarks using the NIN methods

With the exception of the thermal paper all of the samples were immersed in ninhydrin, allowing the solvent to evaporate before placing them into open A4 plastic

protector sheets for development in the dark [109]. The ninhydrin thermal receipt paper samples were treated by applying the same dry fuming method used on the DMAB samples. This involved placing the replicate between two ninhydrin treatment papers for at least 2 days.

2.2.7 Development of latent fingermarks using ORO

Samples were placed in a glass tray and immersed in the ORO reagent for 15 minutes, with manual agitation provided by gently rocking the tray for 30 seconds at the beginning of treatment, according to Frick *et al.* [169]. After development, ORO treated samples were rinsed twice in a deionised water bath under running water, and air dried on paper towels at room temperature.

2.2.8 Development of latent fingermarks using PD

Apart from one minor modification, where the maleic acid pre-treatment step was increased from 5 minutes to 30 minutes as recommended by Salama *et al.*, the procedure used was as described by the National Centre for Forensic Studies [109, 171]. Samples were rinsed in deionised water for 10 minutes, immersed in maleic acid for 30 minutes, and then rinsed again in deionised water for 10 minutes. They were then submerged into the working solution for up to 20 minutes. After development was achieved, samples were rinsed several times in deionised water and air dried on paper towels at room temperature away from direct light. Each step was carried out in a separate glass tray.

2.2.9 Optimisation of the wet contact DMAB reagent

Various solvents (acetone, ethanol, ethyl acetate, HFE-7100, methanol, petroleum spirits, propylene glycol) were trialled to evaluate not only the fingermark development, but also safety, destruction of evidence and its effect on the reagent in sequence with other treatment options. A range of DMAB concentrations (0.4, 0.8, 1.2, 2.4 and 4.8 g/100 mL) were tested to find the optimum working solution. In addition, numerous acids (citric, glacial acetic, maleic, nitric, sulfuric and hydrochloric acids) at varying concentrations (0.018-2.5 M) were investigated.

The effects of heat to aid the fingermark development were investigated using an Elna laundry press (10, 20, 30, 45 and 60 seconds at low, medium or high (~160 °C) temperature settings) and an oven (10, 20 and 30 minutes at 75, 100, 125, 150 or 175 °C). A brief preliminary study into using a Foster & Freeman Thermal Fingerprint Developer (TFD-2) (40, 60, 80 and 100 % heat and at a tray speed of 1500 or 3000 mm/min), which equates to a paper surface temperature of between 100-200 °C, was also tested. The contact time was varied from a range 1, 2-3 and 10 seconds, as well as 5 and 10 minutes.

2.2.10 Optimisation of the dry contact DMAB method

Solvent investigations included acetone, ethanol and ethyl acetate. Concentration studies consisted of 1, 2, 4 and 6 g of DMAB dissolved in 100 mL of ethyl acetate. Acetic, citric and sulfuric acid were also trialled. The dry contact treatment papers were prepared by dipping white copy paper into the working solution and allowing the sheets to air dry before storing in a sealed zip-lock plastic bag. As above, the effects of heat to aid the fingermark development were investigated using an Elna laundry press (20, 30, 45 & 60 seconds at the high (~160 °C) temperature setting) and an oven (30 & 60 minutes and 1 & 5 days at 50 & 80 °C as well as 150 °C for 20 minutes).

2.2.11 Robustness of the dry contact DMAB treatment method

The robustness of the DMAB treatment method was evaluated by changing the following parameters. The concentration of DMAB was varied from 1, 2, 4 and 6 g per 100 mL of solvent. The contact time was varied from 2, 4, 5, 7, 10, 15, 20, 25 and 30 days. A reagent solution (consisting of 4 g DMAB in 100 mL ethyl acetate) was stored in a darkened flammables cabinet for 12 months. Sample degradation was evaluated by storing treated samples in a temperature controlled laboratory within a darkened cupboard. Treatment papers were made fresh, and stored for 0, 5, 10, 15, 20, 25 and 30 days prior to sample treatment to evaluate their stability.

2.2.12 Dry contact DMAB environmental conditions

Ten donors on three different occasions deposited fingermarks on white copy paper. Each of the three replicates was halved and separated for treatment with DMAB or

ninhydrin and further divided into controlled and semi-controlled environment groups to be aged 2, 4 and 6 weeks prior to development. The controlled conditions present from the collection date through to development were a near constant temperature of approximately 24 °C and a maintained relative humidity of approximately 50 % in darkness. The climatic extremes measured by the Bureau of Meteorology, Australia, at the semi-controlled storage were 22.0-41.5 °C and a relative humidity range of 20 – 96 % [172, 173]. Similar minimum temperatures were observed within the storage location; however, the daily maximums were about 10 °C warmer.

2.2.13 Amino acid spot tests

Solutions of l-serine, l-alanine and glycine were prepared in water at concentrations of 15 µg/µL and 0.15 µg/µL. The amino acid spot tests were created by dispensing 10 µL onto white copy paper and allowed to air dry in a darkened cupboard. These spots were then treated with the fingermark reagents as per the recommended method.

2.2.14 Luminescence spectrophotometry

Solutions of alanine, serine and glycine in water (at 15 µg/µL (high) and 0.15 µg/µL (low) concentrations) were used to investigate the luminescence characteristics of DMAB. 10 µL spots on paper were air dried and then subjected to DMAB development as per the method for latent fingermarks. Luminescence spectra were obtained using a Cary Eclipse Fluorescence Spectrophotometer with a fibre optic probe attachment (Varian, Mulgrave, Australia). Data was recorded as an average of 10 scans and with excitation and emission slit widths of 5 nm.

2.2.15 Photography of samples

Samples were photographed using a Nikon D300 camera, equipped with an AF-S Micro-Nikkor lens, mounted on a Firenze Mini Repro tripod and connected to a computer using Nikon's Camera Control Pro Version 2.0.0. Illumination in luminescence mode was achieved using a Rofin Polilight® PL500 (Rofin, Australia), with an excitation wavelength of 490 nm (and 505 nm for IND comparisons, 40 nm bandwidth) and an orange camera filter attachment (Foster + Freeman Schott

OG550). A 3 W Cree® Blue LED Mini Spot Light was used as an alternative excitation source. Illumination in absorbance mode was achieved using incandescent light bulbs with no camera filter attachments. Table 2.2 gives a summary of the photographic conditions. Later adjustments of the images were performed on Adobe Photoshop CS5 Version 12.1, only for clarity of the figures in this article. Evaluation of the fingermark development was carried out on raw images.

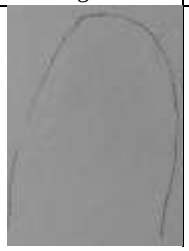
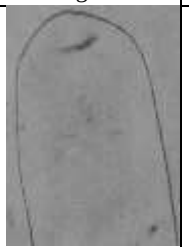
Table 2.2 Photographic conditions for absorbance and luminescence mode photographs.

	Absorbance mode	**Luminescence mode**
Focal Length/mm	60	60
Exposure Mode	Manual	Manual
White Balance	Auto	Auto
Shutter Speed/s	1/20	1
Aperture	f/11	f/11
Sensitivity	ISO 200	ISO 200

2.2.16 Visual analysis of developed latent fingermarks

Treated fingermarks were graded using a 5-point system based on that used by the Home Office Police Scientific Development Branch (HOPSDB) seen in Table 2.3 [174]. Later adjustments of the images were performed on Adobe Photoshop CS5 Version 12.1.

Table 2.3 Grading system for developed latent fingermarks based on the HOPSDB scale [174].

Grade	**0**	**1**	**2**	**3**	**4**
Friction Ridge Detail Development	No development	Signs of contact, but less than 1/3 of fingermark continuous ridges	1/3 – 2/3 of fingermark continuous ridges	More than 2/3 of fingermark continuous ridges, but not quite a 'perfect' fingermark	Full development; whole fingermark, continuous ridges
Background Development	Heavy background	Heavy background	Medium background	Very light background	No background
Photographic Representation					

2.3 Results and discussion

2.3.1 Wet contact DMAB reagent

Ehrlich's reagent, for which many formulations exist but most contain DMAB dissolved in ethanol, is widely used in histochemical studies [158]. To ascertain whether DMAB had any potential as a fingermark detection reagent, samples on paper were treated with an Ehrlich's reagent formulation of 1 g DMAB in 100 mL ethanol and heated for 10 seconds in an Elna laundry press. Faint brown impressions were observed which were weakly photoluminescent when illuminated at 490 nm and viewed through an orange filter (OG550), indicating that DMAB was reacting with the fingermark deposits on the paper.

2.3.1.1 Method development

Typically, latent fingermarks on paper are developed by immersing the paper exhibit into a solution of the relevant reagent, then drying by evaporation of the solvent, or in some cases heating [7, 101, 155]. For amino acid sensitive reagents a wide variety of solvents have been used, with an ideal solvent being volatile (so as to facilitate drying time), non-toxic and non-polar. This last feature is important to avoid the running of ink on documents [107]. In addition, co-solvents may be required to assist dissolution of the reagent in the non-polar carrier solvent. The presence of acids and metal salts have also been shown to improve performance for some treatments [6].

A variety of solvents that have been applied to fingermark reagent formulations (acetone, ethanol, ethyl acetate, HFE-7100, methanol, petroleum spirits (40-60 °C and 60–80 °C) and propylene glycol) were investigated for their performance in relation to fingermark development, minimisation of damage to the exhibit, and the effect on the sequencing with other treatment options. A range of DMAB concentrations (0.4, 0.8, 1.2, 2.4 and 4.8 g/100 mL) and a variety of acids (citric, glacial acetic, maleic, nitric, sulfuric and hydrochloric acids) at varying concentrations (0.018-2.5 M) were examined in combination with the carrier solvent. It was found that the choice of acid had a much greater effect than the choice of solvent. For example, although acetone and ethanol were inferior to methanol and ethyl acetate, some fingermark development could still be obtained. However, using

an unsuitable acid (such as sulfuric, maleic or citric acid) resulted in very faint or no development at all. It was found that a formulation consisting of DMAB in ethyl acetate and glacial acetic acid gave yellow brown fingermarks (Figure 2.4) which exhibited photoluminescence when illuminated at 490 nm with a high intensity filtered light source and viewed through an orange filter (OG550).

Figure 2.4 Fingermark treated with DMAB working solution. Photograph taken with a Nikon D300 camera in absorbance mode; focal length: 60 mm, shutter speed: 1/20 second and aperture: f/11.

However, a formulation based solely on ethyl acetate as the carrier would be unsuitable for operational use as this would cause issues with the running of ink on written documents. DMAB is insoluble in non-polar solvents (such as petroleum spirits and HFE-7100); however, it was found that a stock solution of DMAB in ethyl acetate and acetic acid could be added to HFE-7100 to yield a working solution to successfully develop latent fingermarks (Figure 2.5). The ratio of DMAB: acid: ethyl acetate was adjusted to improve the response of the reagent in combination with HFE-7100. The concentration of DMAB investigated ranged from 0.2 to 1 g per 100 mL of solvent, with little variation seen in development quality. The concentration of DMAB in Ehrlich's Reagent ranges from 0.08 to 1 g per 100 mL. Hence, it was not surprising to find that the different trialled DMAB concentrations had little effect on the overall fingermark development [158, 160].

Figure 2.5 Fingermark treated with (a) 1 mL of stock solution (1 g DMAB in 22 mL ethyl acetate and 0.5 mL acetic acid) in 9 mL HFE-7100 and (b) 0.6 g DMAB in 49.5 mL ethyl acetate and 0.5 mL acetic acid. Photograph taken with a Nikon D300 camera in luminescence mode; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

The formulation that provided the best results was determined to be a final wet contact working solution of 1 mL of stock solution (1 g DMAB in 22 mL ethyl acetate and 3 mL acetic acid) in 9 mL HFE-7100. The working solution gave satisfactory fingermark development after one month of storage, with little or no decrease in reagent performance. However, as DMAB is degraded by UV radiation, the stock solution must be stored in a darkened cabinet, in a tinted storage bottle and/or by wrapping aluminium foil around the storage bottle.

Having the optimal reagent formulation does not necessarily result in good fingermark development. Two very important aspects in the treatment stage are the contact time of the reagent with the sample and, if necessary, the application of heat to increase the reaction rate. Some reagents require long contact times to ensure that the complete reaction or staining can take place. However, the longer the contact time, the greater the possibility of removing parts of the fingermark deposits and/or damaging the exhibit, while possibly not offering significant improvement to the development. This is especially important if the reagent is to be used in sequence with other treatment options that may target components that could be washed away by the solvent. For example, ORO has been shown to be affected by the length of contact time, whereas PD appears unaffected [175]. The contact time of the sample with the working solution was varied, with a range of 1, 2-3 and 10 seconds, as well as 5 and 10 minutes, being trialled. Dipping the samples into the working solution for around 1-2 seconds appeared to offer the best compromise when considering the above factors.

The effects of heat to aid the fingermark development were investigated using an Elna laundry press (10, 20, 30, 45 and 60 seconds at low, medium or high (~160 °C) temperature settings) and an oven (10, 20 or 30 minutes at 75, 100, 125, 150 or 175 °C). In the case of the Elna press, heating the treated samples for 20 seconds at ~160 °C provided the best balance of developing the complex and protecting the paper from charring. Very poor development resulted at lower temperature settings, while shorter heating times (10 seconds) resulted in underdevelopment and long heating times (30 seconds) resulted in the charring of the paper.

For samples placed in an oven, the best balance between development (not occurring at temperatures of 75-125 °C) and non-charring of the paper (which occurred at 175 °C) was found to be at 150 °C for 20 minutes. The results obtained with the oven were comparable to those obtained with the Elna press. The oven was more likely to over-develop fingermark samples and give more background development; however, it appeared to be slightly more effective for weakly developed samples (Figure 2.6).

Figure 2.6 Fingermark treated with DMAB working solution, (a) heated in an oven and (b) an Elna laundry press. Photograph taken with a Nikon D300 camera in luminescence mode; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

2.3.1.2 Photoluminescence studies

In order to investigate whether the DMAB was reacting with the amino acid content of latent fingermark deposits, solutions of amino acids in water (alanine, serine and glycine, at 15 µg/µL (high) and 0.15 µg/µL (low) concentrations) were deposited (10 µL) on paper, dried and then subjected to DMAB development as per latent

fingermark treatment. These amino acids were selected as they have been reported as being amongst the most abundant amino acids in latent fingermarks [24, 55].

All of the treated high concentration spots appeared as yellow-brown impressions which exhibited strong photoluminescence when illuminated at 490 nm and viewed through orange filter (Figure 2.7). The colour and luminescence intensity was demonstrated to be much lower for the low concentration spots.

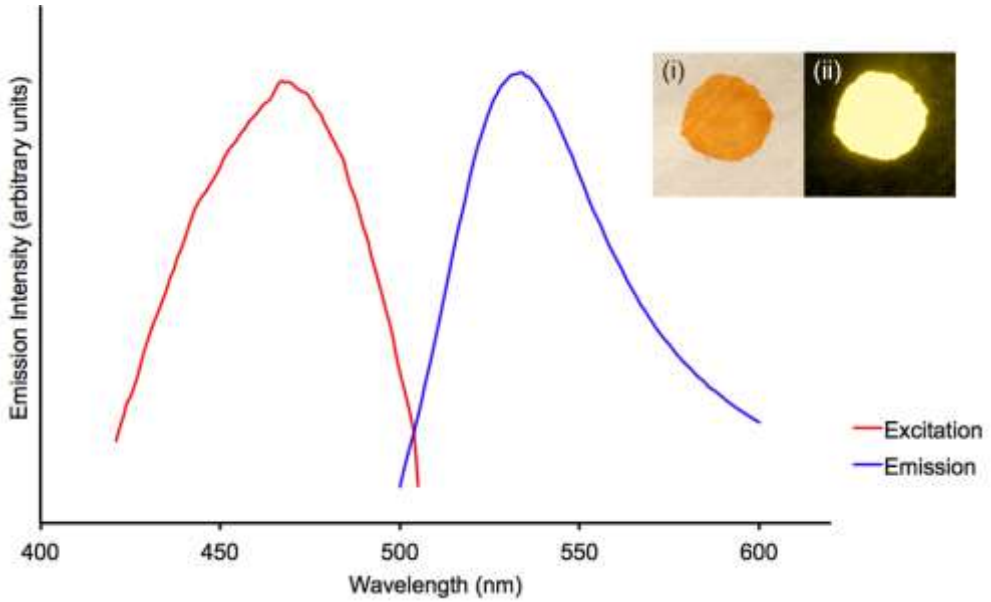


Figure 2.7 Luminescence spectra for L-alanine amino acid spots on paper developed with DMAB. Inset image of developed L-alanine spot on paper photographed by Nikon D300 camera; focal length: 60 mm, in (i) absorbance mode; shutter speed: 1/20 second and aperture: f/11 (ii) luminescence mode; shutter speed: 1 second and aperture: f/11

Photoluminescence excitation and emission spectra for the treated high concentration alanine spot are presented in Figure 2.7 and the emission spectra of each of the treated amino acid spots and a treated latent fingermark are presented in Figure 2.8. Each of the treated amino acids had a similar excitation wavelength response (at 530 nm) of 480 nm. Emission spectra for the treated amino acid spots and latent fingermark were collected using an excitation of 490 nm (as this matches the available wavelength on the Polilight). The emission spectra presented in Figure 2.8 show similar maxima, except for l-alanine, which is shifted by around 15 nm towards the blue. This is not surprising as each amino acid would result in a different imine (Figure 2.2). The values for emission and excitation wavelengths for the DMAB developed amino acids are similar to the values observed by Khalil (excitation at

~475 nm) and Cessi and Piliego (emission at 545 nm), although they used different target nitrogenous compounds [160, 176]. The emission spectrum for the developed latent fingermark is very similar to developed amino acids spots indicating that the DMAB is targeting the amino acids in the latent fingermark deposit. The marginal increase in width in the emission peak for the DMAB developed latent fingermark is likely to be due to the range of amino acids in the fingermark and their relative concentrations.

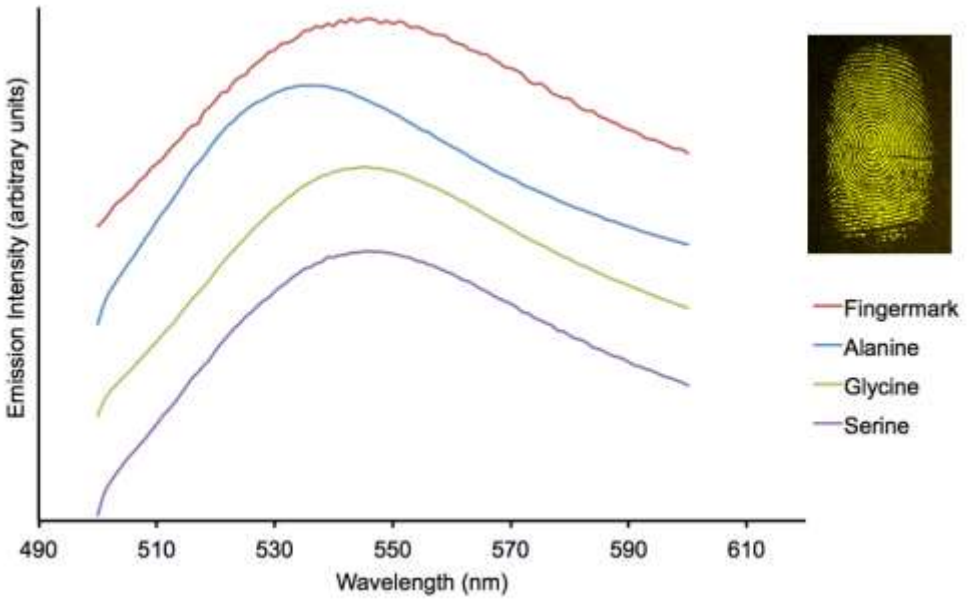


Figure 2.8 Luminescence spectra for amino acid spots (L-alanine, glycine and L-serine) and latent fingermark on paper developed with DMAB. L-alanine, glycine, L-serine spot tests, as well as a developed latent fingermark (photograph). Spectra have been normalised and offset to illustrate similarities and differences in shape and maxima. Inset photograph taken with a Nikon D300 camera in luminescence mode; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

From an operational point of view, more than 90 % of the excitation and emission is in the range from 473-495 and 527-547 nm, respectively. This corresponds to using a blue-green light source, such as available with the Polilight, with an orange barrier filter and is in fact very similar (and should require no extra instrumentation) to the conditions used for IND treated fingermarks. Although Takatsu *et al.* indicate that the best visualisation occurs when exciting with a UV-source (at 365 nm), due to the optical brighteners used in nearly all paper sources (which make the paper appear more 'white'), this will give rise to a very bright background, greatly reducing the contrast.

2.3.1.3 Sequencing with other fingermark development methods

It is possible to increase the number of successfully developed fingermarks by treating the samples in sequence with different development methods, especially when handling challenging exhibits [6]. This is typically performed using one or two amino acid sensitive reagents, followed by reagents targeting the sebaceous secretions [109]. This approach not only allows for a greater variety of substrates to be treated, but it is also effective in treating samples that have been subjected to water. DMAB was therefore tested in sequence with other latent fingermark development reagents, where samples were treated with either DMAB or IND/$ZnCl_2$, followed by ORO and PD (Figure 2.9). This sequence also allowed the sensitivity of DMAB to be compared to an amino acid sensitive reagent in current operational use (IND/$ZnCl_2$).

It was found that the performance (i.e. luminescence) on strong fingermark impressions is similar with IND/$ZnCl_2$ or DMAB; however, IND/$ZnCl_2$ provides better visualisation on weak prints. It was observed though that ORO performs much better in sequence on DMAB treated fingermarks, with improved colouration and more detail visible when compared to IND/$ZnCl_2$. It should be noted that it was found in a previous study that the exposure to non-polar solvents has to be limited to prevent lipid migration to enable the successful application of the ORO reagent [175]. More interestingly, luminescent detail could still be seen, although diminished, on the DMAB treated fingermark following ORO treatment (Figure 2.10). This would suggest that the imine/enamine complex is water soluble to a lesser extent than the Joullié's Pink complex formed with IND/$ZnCl_2$. Both treated fingermark halves reacted to the same extent with PD, with slightly better development again occurring in sequence with DMAB. As with ORO, fingermarks that were very strongly developed with DMAB still offered luminescent detail after the destructive PD sequence (Figure 2.10).

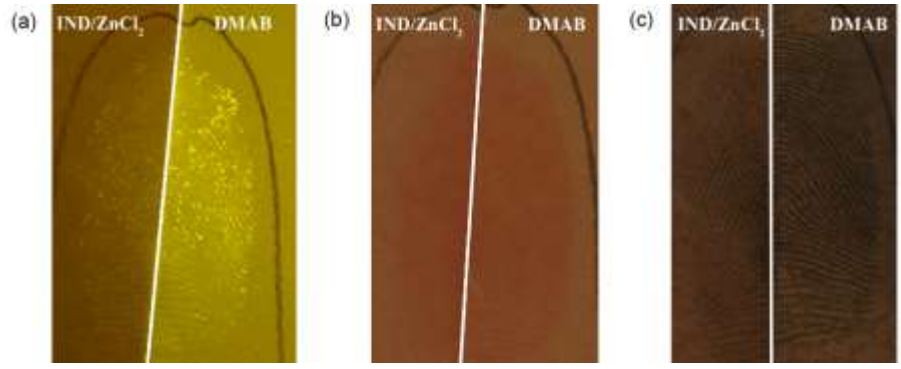


Figure 2.9 Charged fingermark sample treated with (a) IND/$ZnCl_2$ (left-half) or DMAB (right-half), followed by (b) ORO and then (c) PD. Photographs taken with a Nikon D300 camera in luminescence mode (DMAB and IND/$ZnCl_2$); focal length: 60 mm, shutter speed: 1 second and aperture: f/11; and absorbance mode (ORO and PD); focal length: 60 mm, shutter speed: 1/20 second and aperture: f/11.

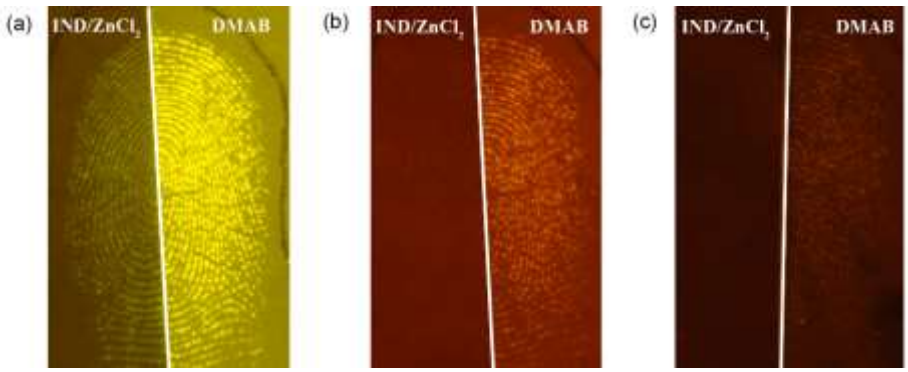


Figure 2.10 Fingermark treated with (a) IND/$ZnCl_2$ (left-half) or DMAB (right-half), followed by (b) ORO and then (c) PD. Photographs taken with a Nikon D300 camera in luminescence mode; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

2.3.1.4 Treatment of aged samples

Latent fingermark deposits are frequently required to be developed after extended periods following deposition. Fingermark deposits that had been left in ambient conditions for extended periods were treated with the final DMAB working method. Interestingly, full detail was still observed in strong fingermarks even after one month; however, all samples showed diminished intensity compared to the halves that were developed when fresh. This is in contrast to Sasson and Almog's work with *p*-dimethylaminocinnamaldehyde (DMAC), where fingermarks older than 72 hours could not be reliably developed due to their appearance as "unresolved stains" [23]. Fingermark samples developed after 2.5 months showed greatly reduced ridge detail, however, there was no blurring of the ridgelines (Figure 2.11) as was the case with Sasson and Almog's DMAC method. It was noted that the intensity of IND/$ZnCl_2$ treated latent fingermarks less affected than DMAB treated samples.

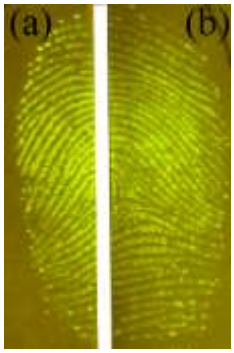


Figure 2.11 One month old fingermark treated with (a) IND/$ZnCl_2$ and (b) DMAB working solution. Photograph taken with a Nikon D300 camera in luminescence mode; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

2.3.2 Dry contact DMAB method

As substrates may be encountered that cannot be subjected directly to either the solvents or acid contained in the working solution, a dry contact DMAB method was also evaluated. This consists of treating blank sheets of white copy paper with the reagent, and then placing the sample in between these dried sheets. This approach was followed and adapted from the IND dry contact process developed by Patton *et al.* [124]. Several factors affect the best possible development of fingermark samples using chemical approaches. In the case of dry contact methods, parameters that may be altered include the composition of the reagent, the contact time and the use of heat to provide energy for a faster reaction. In line with the recommendations provided by IFRG, the dry contact DMAB was evaluated as a larger scale Phase 2 study, which requires the use of a larger donor and substrate pool amongst other factors [100]. This is in contrast to the more preliminary nature of the Phase 1 wet contact DMAB study discussed above. Segments of the dry contact DMAB approach were investigated in collaboration with B. Dorakumbura and B. Hackshaw.

2.3.2.1 Method development

Reagent composition

It is vital to have sufficient DMAB available to fully react with the amino acid target compounds, in order to obtain well-developed ridge detail. Although using an excess of the reagent is not as problematic with dry contact methods as it is with wet contact techniques (where over-development and background staining is more likely to occur), factors such as cost and waste management still have to be considered for

operational use. Concentrations in the range of 1 to 6 g per 100 mL were tested, where no difference to development was found in the higher amounts. Amounts of 1, 2 and 4 g showed similar results in stronger deposits, but overall development was decreased with 1 g (Table 2.4). At the lowest concentration, there was less spotting of the substrate, hypothesised to be from DMAB agglomeration due to the drying process. The spotting of the substrate could be further minimised by leaving the treatment papers to dry for longer periods of time. Although the background was darker and therefore better for contrast at 1 g, ridge detail was minimised and ultimately of less use for operational purposes. Samples split and treated with 2 and 4 g showed no significant difference in ridge detail; however, the background spotting was reduced at 2 g.

Table 2.4 Photographs of fingermark deposits treated with DMAB at 1, 2 or 4 g per 100 mL.

Substrate	4 g (left) vs. 1 g (right)	4 g (left) vs 2 g (right)
White copy paper		
Thermal paper receipts		

In addition to the concentration of DMAB, the type of solvent can have a marked effect on sample integrity and development. Due to the polarity of DMAB, non-polar solvents cannot be used. However, as the exhibits are not dipped directly into the working solution, polar solvents should not adversely affect inks and thermal papers. Acetone, ethanol and ethyl acetate were trialled, where ethanol resulted in less overall development. Ethyl acetate and acetone could be used with no marked

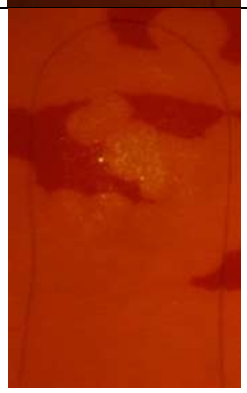
difference in ridge detail. It should be noted that acetone evaporates at a lower temperature (56.2 °C) than ethyl acetate (77 °C) which does mean that the treatment papers dry quicker; however, when preparing multiple treatment papers the working solution also evaporates much more rapidly. The remainder of this study was therefore performed using ethyl acetate.

It was found that the inclusion of any form of acid in the dry contact formulation inhibited the imine/enamine formation [167]. Further work is required to establish why the acidity of the working solution negatively affects the dry contact approach, but positively affects the wet contact DMAB method. The omission of acid does eliminate an extra preparation step and also makes the entire procedure safer and cheaper to use. Therefore, a working solution consisting of 2 g of DMAB per 100 mL ethyl acetate, or acetone, was found to offer a the best development overall. From a health and safety perspective, ethyl acetate is much safer due to its higher flash and boiling points.

Contact time

Fingermark deposits on both white copy paper and thermal receipt paper (on used receipts and fresh thermal receipt roll) showed little further development when photographed every 5 days over a one month period (Table 2.5). Tests indicated that there was little difference between samples kept in contact with the treatment papers for shorter periods, where only marginal improvement to the ridge detail was observed when using prolonged contact in a 2, 4, 5 and 7 day timeframe. For the purposes of this study, specimens were in contact with the treatment papers at room temperature for a point of comparison.

Table 2.5 Photographs of DMAB treated fingermark deposits after extended contact periods.

	10 days	20 days	30 days
White copy paper			
Thermal paper			

Heating

Using a heat source can speed up the imine reaction, and therefore cause sample development to occur much more rapidly. A fingermark reagent should provide complete development as quickly as possible so as to make it more amenable for operational purposes. As dry contact methods are often used for fragile exhibits, using heat may not be practicable in all circumstances. The current method gave very good results by heating samples between treatment papers for 45 seconds in an Elna laundry press at approximately 160 °C. Alternative heating conditions were sought for fragile samples [167].

Exhibits placed in between treatment papers and wrapped in aluminium foil showed improved development compared to samples kept at room temperature for 5 days when heated in an oven at 50 or 80 °C (Table 2.6). After 24 hours most of the ridge detail had developed, and further heating provided little benefit. While thermal substrate degradation was less pronounced with exhibits placed in an oven at 50 or 80 °C compared to the Elna laundry press due to the lower temperatures, the application of heat to speed up the reaction process is still found to be unsuitable for

temperature sensitive samples. For the much quicker and easier treatment of non-fragile samples, the Elna laundry press is still recommended over the oven. Where heat treatment is not feasible, a contact time of 2 days (or until sufficient ridge detail is visible) at room temperature should be used instead.

Table 2.6 Photographs of DMAB treated fingermark deposits, with and without the application of heat.

Substrate	Room temperature (left) vs. 50 °C oven (right)	80 °C oven (left) vs. room temperature (right)
White copy paper		
	50 °C oven (left) vs. room temperature (right)	**80 °C oven (left) vs. room temperature (right)**
Thermal paper receipts		

2.3.1.2 Operational considerations

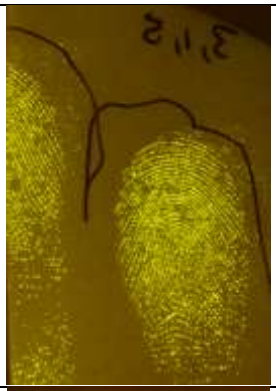
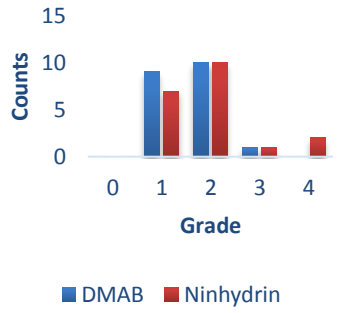
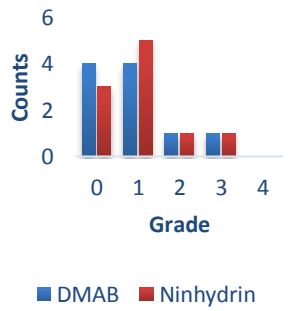
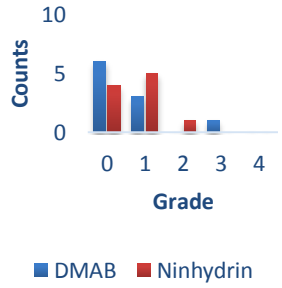
The improved method was applied to a larger range of samples, as the reagent conditions tested for use on copy paper and thermal paper must also afford good development with potentially more difficult exhibits. In addition to the testing of various substrates, the method was also investigated for its robustness and use on older fingermark exhibits. Blind studies and depletion series gave an indication of the reagent's sensitivity in comparison with ninhydrin.

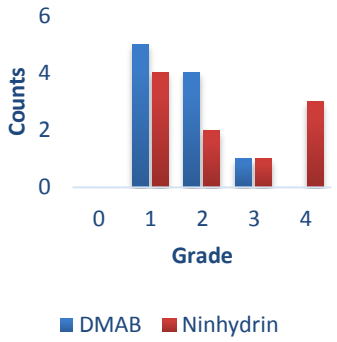
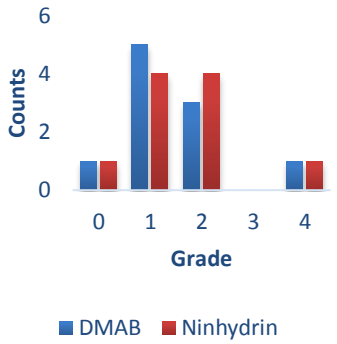
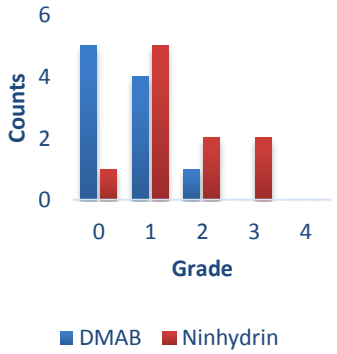
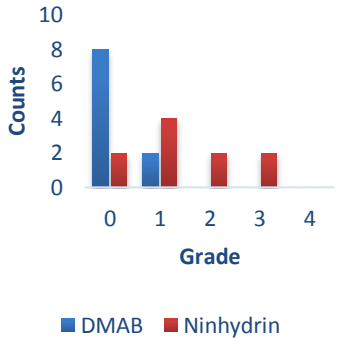
Substrate investigations

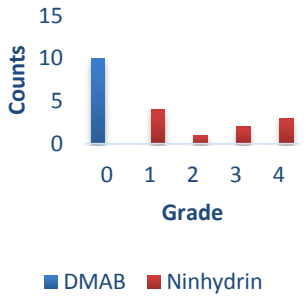
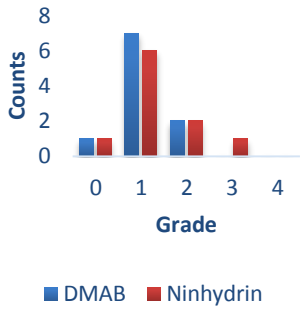
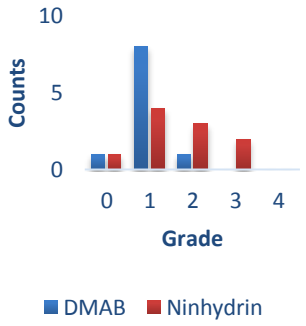
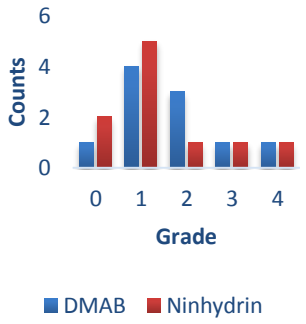
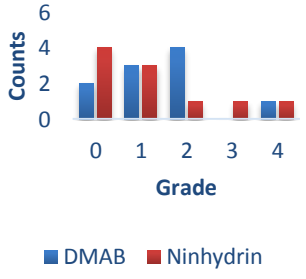
A wide range of different porous and semi-porous substrates are encountered as everyday items, including gift wrapping paper, coloured paper, envelopes, Post-it® notes, thermal paper, filter paper and newspaper. In this study, the response of latent

fingermarks deposited on a number of these porous and semi porous substrates and treated with DMAB were compared to ninhydrin. The purpose of this was not to establish which of these methods was more sensitive, but rather to determine the efficacy of DMAB on a range of exhibits. Ninhydrin was used as an indication of strong or weak deposits (Table 2.7). In these investigations it was deemed appropriate to use a fingermark grading scale adapted from Bandey *et al.*, which assesses the clarity of the ridge detail and contrast to give a measure of reagent performance (as described in Table 2.3) [174]. The grade distribution is also shown for each substrate, giving an indication of the spread of grades given for ninhydrin and DMAB treated samples as a function of absolute counts of grades given.

Table 2.7 Photographs of ninhydrin and DMAB treated fingermark deposits on various substrates and the grade distribution given to both reagents as a function of absolute counts.

Substrate	Ninhydrin treated samples	DMAB treated samples	Grade distribution
White copy paper			
Thermal paper receipts			
Newspaper			

Substrate	Ninhydrin treated samples	DMAB treated samples	Grade distribution
Coloured paper			
Notebook paper			
Orange envelope – inside			
Orange envelope – outside			

Substrate	Ninhydrin treated samples	DMAB treated samples	Grade distribution
Post-it notes			
White envelope - inside			
White envelope – outside			
Wrapping paper - inside			
Wrapping paper - outside			

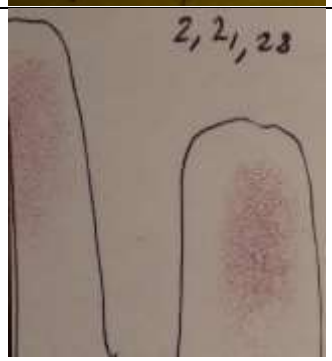
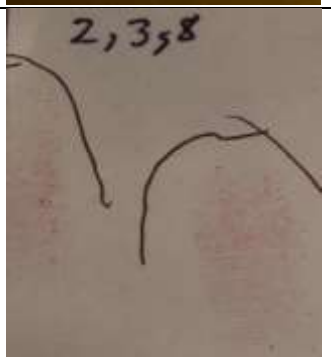
The DMAB dry contact method works better on glossy, bright white and smooth surfaces (for example wrapping paper and white copy paper). It is likely that the improved performance on less porous substrates occurred due to enhanced surface area contact with the treatment papers on these smoother surfaces.

Aged prints and environmental conditions

One aspect highlighted by Kent [145], as well as the IFRG [100], is that method optimisation and comparison studies should consider aged fingermarks as well as relatively fresh deposits. To investigate the effect of time since deposition, fingermark specimens on white copy paper that were 6 and 18 months old were treated. Ninhydrin was again used as a point of reference, yielding samples which were less developed than the dry contact DMAB halves. Out of the fingermark deposits investigated, ninhydrin achieved a mean grade of 0.5 compared to 0.8 for DMAB (median grade of 0 and 1, respectively).

In addition, a trial was conducted to simulate the effects of environmental factors that forensic exhibits may be subjected to prior to collection and/or treatment. Samples that had been deposited on white copy paper and left at controlled (within a climate regulated laboratory) and semi controlled (under cover outside) locations for 2, 4 and 6 weeks prior to treatment showed significant differences between ninhydrin and DMAB. Ninhydrin produced development on nearly all of the samples for each of the time periods and environments. A gradual downward trend in ninhydrin development was observed mainly in the semi controlled environment which had experienced some extreme tropical weather (from a mean grade of 2.1 to 1.7). The mean grade remained constant for specimens stored in a controlled environment. Many of the 2 week old DMAB treated fingermarks displayed development (mean grade of 1.3), yet this was found to be reduced after a further 2 weeks, where a more significant decrease in ridge detail was observed in the semi controlled compared to the controlled samples (1.1 vs. 1.4, respectively). None of the 6 week old specimens showed development using DMAB. Overall, ninhydrin outperformed DMAB as a fingermark reagent for use on deposits aged under harsh environmental conditions (mean grade of 1.9 vs. 0.8), indicating a more sensitive reagent (Table 2.8).

Table 2.8 Photographs of ninhydrin and DMAB treated fingermark deposits on white copy paper and aged for 2, 4 and 6 weeks.

Ageing period	2 weeks	4 weeks	6 weeks
DMAB treated samples			
NIN treated samples			

Sensitivity

As outlined by Kent and the IFRG guidelines, the sensitivity of a reagent can be assessed using a depletion series [100, 145]. While amino acid rich fingermark deposits may give a strong response even to unsatisfactory reagents, due to the decreasing amount that is transferred with each additional deposition, a depletion series can indicate reagent response when approaching the limit of detection [100]. Whilst development was present in nearly all the treated marks, the strength of the ridge contrast was greatly reduced from the second deposit onward. Little difference between ninhydrin and DMAB treated depletion series could be observed.

The IFRG guidelines also recommend blind studies as a good indication of "the ability of the technique to perform on unknown samples" and provides another point of comparison with established methods [100]. Members of this research group have previously used blind studies for the evaluation of a dry contact IND method, and the same approach was therefore implemented in this study [124]. In this case, all of the ninhydrin reference specimens gave 100 % agreement of displayed versus the actual

number of marks deposited. The dry contact DMAB method also gave a success rate of 100 %; however, the very weak deposits were much harder to discern and only very faint impressions could be observed.

Robustness

The robustness of any technique, whether new or altered, is imperative for the routine employment by law enforcement personnel. Ideally, a method should work even if its parameters, such as concentration or contact time, are changed slightly by the user [170]. In this regard, the dry contact DMAB procedure was observed to offer similar levels of development whether a concentration of 2 to 6 g per 100 mL solvent was used and at a range of contact times, from 2 to 30 days.

Other aspects to consider when developing a new/altered reagent formulation is the working shelf life of treated fingermark exhibits, the working reagent solution and the treatment papers (in the case of dry contact methods), and that these are stable for extended periods of time. As it may not always be possible to photograph ridge detail immediately following treatment, it is of importance that the reaction product is lasting. Samples photographed every 5 days over a one month period after treatment indicated that little degradation had occurred. A 12 month old working solution also provided very similar levels of development to the fresh formulation, with no appreciable loss in intensity. Lastly, treatment papers that had been stored in zip-lock bags for 5-30 days once again revealed indistinguishable results to fresh treatment papers. These experiments indicate the dry contact DMAB method to be a robust, practicable technique, amenable to routine implementation.

Samples suitable for identification purposes

The IFRG guidelines recommend for Phase 3 and above that some measure of how many developed marks are suitable for identification [100]. While the study presented here is clearly at an earlier stage in the evaluation process, the suitability for identification of the DMAB treated marks on a variety of substrates was compared to those developed with ninhydrin by a fingerprint expert. The results are summarised in Table 2.9. As can be seen from the data presented, while there may be

some similarity in the mean grades on some substrates, this does not necessarily correspond to suitability for identification. It should be noted though that the grades obtained for both ninhydrin and DMAB are on the low end of the classification scale. Of the substrates tested, only the smooth wrapping paper resulted in more marks being identifiable after DMAB rather than ninhydrin treatment. This was also reflected in the mean and median grades of this substrate, where DMAB was rated higher than ninhydrin. In general, it was noted that there was an overall trend that samples which were given poor grades were less likely to give identifiable marks. Studies are currently underway to investigate the correlation between grading of developed marks with a scale versus a determination of whether they are suitable for identification by a fingerprint expert.

Table 2.9 Absolute counts (where n=10) for samples where identification is and is not possible for DMAB and ninhydrin treated substrates, and their corresponding mean grade.

Substrate	DMAB			Ninhydrin		
	Identifiable		*Mean Grade*	*Identifiable*		*Mean Grade*
	Yes	*No*		*Yes*	*No*	
Coloured paper	6	4	1.6	10	0	2.3
Newspaper	2	8	0.6	3	7	0.7
Note book paper	4	6	1.5	8	2	1.6
Orange envelope - inside	0	10	0.6	7	3	1.5
Orange envelope - outside	1	9	0.2	6	4	1.4
Post-it notes	0	10	0	6	4	2.4
White envelope - inside	0	10	1.1	4	6	1.3
White envelope - outside	2	8	1	7	3	1.6
Wrapping paper - inside	5	5	1.7	5	5	1.4
Wrapping paper – outside	6	4	1.5	5	5	1.2
Thermal paper	3	7	0.9	7	3	1

2.3.2.3 Light source comparison

The illumination and recording of all dry contact DMAB treated samples with both the Rofin Polilight® PL500 and the much cheaper LED light sources presented the chance to compare these systems for a large number of DMAB treated samples. It was generally noted that the LED was sufficient to detect ridge development in all samples that the Polilight could excite, but the overall emission intensity was reduced, which impaired visualisation with weaker deposits. All substrate

investigation samples were graded using these two light sources, where the median grade for the Polilight was 1 and 0 for the LED (mean grade of 1 and 0.6, respectively). This further reinforces the view that on difficult or weak impressions, the LED light source may not sufficiently excite the marks for identification purposes. Results of the blind study also suggest that the Polilight provides better excitation of developed samples, as all 75 deposited marks were observed. However, illumination using the LED light source still made possible the observation of 72 deposits. This indicates that although the marks may be weaker, for nearly all of the deposits it was sufficient to demonstrate that a contact had occurred.

As every fingermark reagent that can cause luminescence of treated fingermarks differs slightly as to the required wavelength to achieve the appropriate excitation of the reaction product, the LED light source should be evaluated on a reagent-by-reagent basis. As expected, the overall excitation is lower than that of the Polilight illuminated samples. The cheap and portable nature of the LED may still be of value not only for smaller volume crime laboratories, but also for teaching and demonstration purposes.

2.4 Conclusions

DMAB was found to be a novel fingermark reagent, yielding impressions on paper surfaces that are both coloured and photoluminescent. The wet contact method proved effective on non-fragile porous substrates such as white copy paper and various other substrates. The final wet contact working method consisted of 1 mL of stock solution (1 g DMAB in 22 mL ethyl acetate and 3 mL acetic acid) in 9 mL HFE-7100. The sample is then immersed into the working solution for 1-2 seconds before being air dried on paper towels at room temperature, followed by heating in an oven at 150 °C for 20 minutes. Photoluminescence is observed by illumination with a high intensity filtered light source at 490 nm and viewing through an orange barrier filter (OG550). These preliminary results suggest that IND/$ZnCl_2$ is still the more sensitive technique; however, wet contact DMAB may have some advantages when used in sequence with treatments targeting the sebaceous component of latent fingermark deposits.

Dry contact DMAB was shown to develop latent fingermark deposits on a range of different substrates. It was found that in the current form, the dry contact DMAB approach does not offer the same level of development as ninhydrin and cannot be recommended for routine operational use. The experiments were planned and conducted according to the IFRG guidelines. These guidelines were invaluable in addressing the issues associated with, and presenting the content expected from, a Phase 2 study appropriately.

An alternative excitation source in the form of a LED light was evaluated and compared to a Rofin Polilight®, where the latter's more intense illumination provided better contrast. However, the robust and much cheaper LED light source may be useful in teaching or remote environments where portability is an issue and the expense of the Polilight cannot be justified. The use of the LED light will be further investigated for the application with a range of different reagents, such as IND/$ZnCl_2$.

Chapter 3

A new *p*-dimethylaminocinnamaldehyde reagent formulation for the detection of latent fingermarks by photoluminescence

3.1 Introduction

First studied in 1973 by Morris and Goode, *p*-dimethylaminocinnamaldehyde (DMAC, Figure 3.1) was a novel reagent that was thought to show promise as a ninhydrin alternative [139]. However, an in depth study conducted by Sasson and Almog found that the method was inferior to ninhydrin on nearly all fingermark samples tested [23]. A significant issue encountered was that fingermarks that were treated 72 hours after deposition appeared blurred. This was thought to be due to urea (the proposed target compound) migrating rapidly through porous substrates [23, 140]. It has since been established that DMAC does not exclusively target urea in fingermark deposits; it also reacts with primary and secondary amines, including amino acids [23, 140].

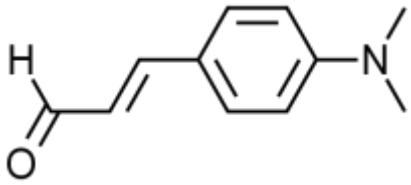


Figure 3.1 Chemical structure of DMAC.

Brennan *et al.* investigated DMAC as a fuming agent, achieving good results on a wide variety of substrates [141]. Fuming was accomplished by passing the sample over heated DMAC and then conditioning overnight at 200 °C at 30 % relative humidity. Substrates treated included aluminium cans, thermal paper, cardboard, polythene and glass. It was further found that this method could be used without detrimental effect in sequence with 1,8-diazafluoren-9-one (DFO), ninhydrin (NIN) and physical developer (PD). In additional research, it was concluded that fuming was less effective than using existing processes, except for the treatment of thermal papers [141].

Ramotowski further pursued DMAC initially as a wet contact reagent, then as a dry contact method [142]. It was found that, as with the method by Sasson and Almog, red coloured ridge detail could be observed with moderate fluorescence using a wet contact approach. However, within several hours the background developed further, giving undefined ridge detail. The vapour contact method, which entailed dipping two treatment papers into the working solution and placing the sample in direct

contact with them, offered light yellow samples with significant luminescence. By reducing the concentration and contact time, improved contrast was achieved. Lee *et al.* further investigated the use of DMAC as a dry contact method on thermal paper such as receipts and facsimile paper [143]. It was found that DFO and NIN were vastly superior techniques to DMAC, with 100 % and 50 % more ridge detail or marks developed, respectively. In addition, amino acid, urea, and sodium chloride spot tests were prepared, treated with DMAC and their excitation and emission spectra compared to treated latent fingermarks. It was found that the spectra for the amino acids most closely resembled those of a latent fingermark, and it is thought that they are the most likely the dominant target compounds for the DMAC reaction.

3.1.1 Reaction pathway

As can be inferred from the structural similarity of DMAC to *p*-dimethylaminobenzaldehyde (DMAB), they undergo the same general reaction mechanism, as described in Chapter 2 (Figure 3.2). A structural advantage of DMAC over DMAB is that it is a longer conjugated molecule. In the push-pull system described in Chapter 2, DMAC should give stronger luminescence and colour due its extra conjugation. As such, it was seen as a logical continuation of the DMAB work to produce a more sensitive reagent. In past studies, it has been noted that DMAC reacts with primary and secondary amines to produce blue and purple Schiff bases, respectively [163].

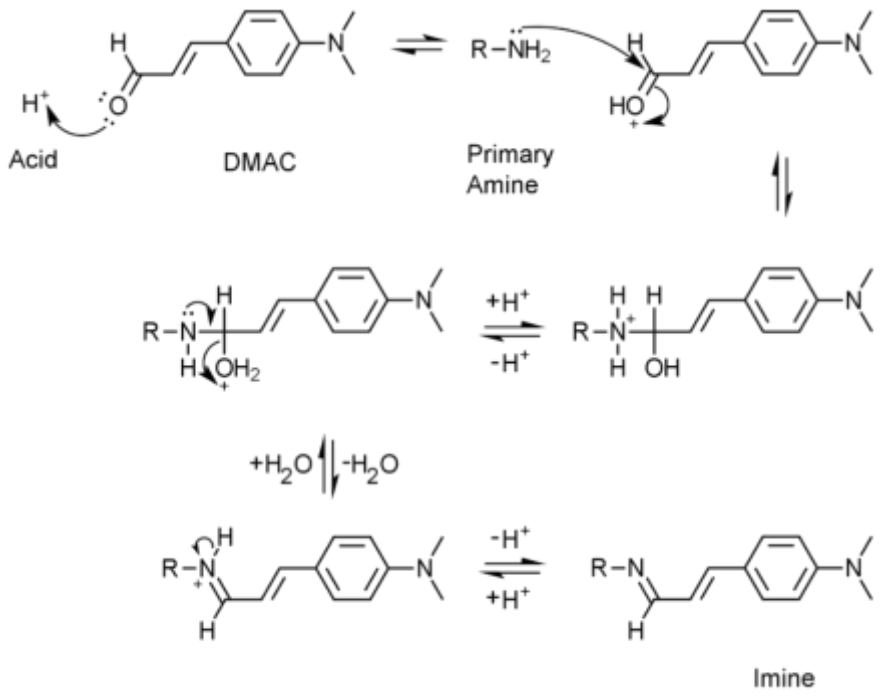


Figure 3.2 General reaction mechanism for the imine formation from primary amines and DMAC (adapted from Adegoke and Nwoke, 2008) [166].

3.1.2 Aims

This Chapter presents the preliminary studies (Phase 1, as recommended by the International Fingerprint Research Group (IFRG) [100]) into the application of a new DMAC formulation as a reagent for the detection of latent fingermarks on paper surfaces. The investigation of a new and improved wet contact DMAC method is set out below, as previous studies have focussed more on the dry contact and fuming approaches to the application of DMAC to latent fingermarks. Its photoluminescent properties were also explored.

3.2 Materials and methods

3.2.1 Chemicals

The following chemicals were used in addition to those listed in Chapter 2.2.1.

p-Dimethylaminocinnamaldehyde (Merck Ltd., Australia), 5-sulfosalicylic acid (Sigma-Aldrich, USA) and urea (Merck Ltd., Australia) were all used as received and were of analytical reagent grade unless otherwise stated.

3.2.2 Substrates

See section 2.2.2 for a list of all substrates used in this thesis.

3.2.3 Collection of latent fingermarks

Refer to section 2.2.3 for the method of fingermark collection used in this thesis. At least 2-4 fingermarks were collected from each donor for each experiment in this chapter.

3.2.4 Preparation of reagent solutions

The preparation of all stock and working solutions of fingermark reagents used in this chapter are summarised in Table 3.1. For conditions pertaining to the use of acidic 1,2-indanedione/zinc chloride (IND/$ZnCl_2$), please refer to section 2.2.4.

Table 3.1 Preparation of the final DMAC stock and working solutions.

Method	Solution	Reagent preparation
DMAC	*DMAC stock solution*	1 mL of 0.02 g DMAC in 4.4 mL ethyl acetate and 0.6 mL acetic acid, added to 9 mL petroleum spirits 40-60 °C
	Working solution	0.7 mL stock solution in 9.3 mL petroleum spirits 40-60 °C
Acid free 1,2-indanedione-zinc chloride [109]	*IND stock solution*	0.35 g IND dissolved in 40 mL ethyl acetate and 960 mL HFE-7100
	Zinc chloride stock solution	8 g $ZnCl_2$ dissolved in 200 mL absolute ethanol
	Working solution	2 mL $ZnCl_2$ stock solution per 1 L of IND stock solution
Dry contact DMAC by Lee *et al.* [143]	*Working solution*	0.25 g DMAC dissolved in 100 mL ethanol
DMAC by Ramotowski [142]	*Working solution*	1 g sulfosalicylic acid dissolved in 50 mL ethanol, added to 0.25 DMAC dissolved in 50 mL ethanol

3.2.5 Development of latent fingermarks using IND methods

The acid- and heat-free IND/$ZnCl_2$ method was performed as described by the National Centre for Forensic Studies [109]. Samples were developed by being briefly dipped in the working solution, and allowed to air dry before storing in the dark at room temperature for 48 hours.

3.2.6 Development samples using established DMAC methods

For the dry contact method proposed by Lee *et al.*, white copy paper (Fuji Xerox Professional, 80 g/m^2) was dipped into the working solution and allowed to air dry [143]. Samples were subsequently sandwiched between two treatment papers, weighted down and stored in the dark for 24 hours.

Ramotowski's wet contact method was used as described, where samples were briefly dipped into the working solution and allowed to air dry [142]. The dry contact method was used in the same fashion as Lee *et al.* above [143].

3.2.7 Optimisation of the DMAC fingermark reagent

Due to the structural similarity of DMAB and DMAC, the DMAB formulation was used as a starting point. Different parameters were changed and adapted to suit the purpose of fingermark identification using DMAC. This included the evaluation of various solvents (ethanol, ethyl acetate, HFE-7100, petroleum spirits (40-60 °C)). A range of DMAC concentrations (0.004, 0.028, 0.04 and 0.4 g/L), and two different acids (glacial acetic and sulfosalicylic acid) at varying concentrations (0.0021-1.89 M) were also examined.

The effects of heat to aid the fingermark development were investigated using an Elna laundry press (10, 20, 30, 60 and 120 seconds at medium or high (~160 °C) temperature settings). This was compared to development without heat, by leaving treated fingermarks in ambient conditions for 15 minutes, and 1, 2 and 3.5 hours).

3.2.8 Other experimental conditions

For the experimental conditions pertaining to the fluorescence spectrophotometry experiments, the photography of samples and the grading of treated fingermarks, please refer to Chapter 2, sections 2.13 to 2.16.

3.3 Results and discussion

3.3.1 Method development

Initial formulation investigations were performed by simply substituting DMAC for DMAB in the final wet contact formulation of DMAB proposed in Chapter 2 (1 mL of stock solution (1 g DMAB in 22 mL ethyl acetate and 3 mL acetic acid) in 9 mL HFE-7100). This produced poor contrast because of intense background colouration and fluorescence. Subsequently, the concentration of DMAC was reduced until more satisfactory contrast was achieved (0.028 g/L). DMAC also requires a polar co-solvent in order to dissolve prior to the addition of the non-polar solvents in the working solution. Both ethanol and ethyl acetate were found to be amenable to this purpose. To enable fingermark development on thermal paper and to prevent inks from running, non-polar solvents should be the main constituent of the working solution. While either petroleum spirits or HFE-7100 was used with no significant variation in performance (Figure 3.3), HFE-7100 is a non-flammable and considerably safer alternative to the much cheaper petroleum spirits.

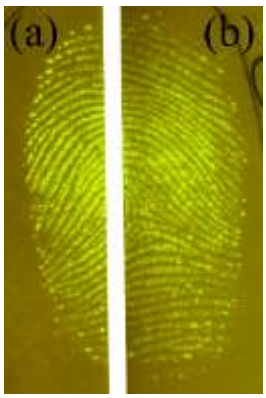


Figure 3.3 Luminescence displayed by a fingermark deposited on white copy paper, treated with a DMAC formulation using HFE-7100 (a) or petroleum spirits (b) as the solvent. Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

The concentration of the acid appeared to have little effect on the overall ridge detail. Increasing or decreasing the acid concentration compared to the final working solution (as well as the absence of acid), only resulted in a relatively minor loss of sensitivity. As DMAC can also be used as an acid-base indicator (red in acidic conditions and yellow in basic conditions, relative to its pKa value of 3.18), the working solution changed colour depending on the strength of the acid used [165].

Various heating methods and intensities were evaluated, where it was found that using an Elna laundry press on a high setting (approximately 160 °C) for 20 seconds gave the best results. For a very similar level of development, the treated samples could also be left for 2-3 hours in ambient conditions without detriment, which is especially useful for fragile or thermal paper samples (Figure 3.4). The dipping time was kept to 1-2 seconds in order to minimise the dissolution of lipids and therefore reduce the impact on lipid-sensitive reagents for possible use in sequence [175].

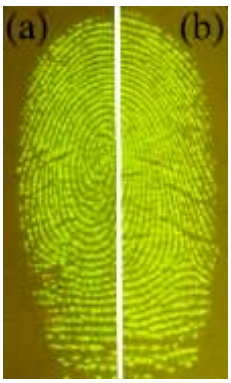


Figure 3.4 DMAC treated fingermark deposited on white copy paper, developed without heating (after 2 hours, (a)) and with Elna laundry press heating (20 seconds, (b)). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

3.3.2 Comparison to 1,2-indanedione/zinc chloride

As IND/$ZnCl_2$ is the main fluorescent amino acid sensitive reagent used in Australia, it was employed as a reference point for the effectiveness of DMAC. The same fingermark grading scale was used as per section 2.2.16, and the relevant mean and median grades are shown below. As expected, IND/$ZnCl_2$ offered better development when applied to weak samples (mean=3.0, median=4 vs. mean=2.4, median 2). In this preliminary investigation, a direct comparison to IND/$ZnCl_2$ was not sought, where its performance on weak and strong marks gave a reference point for expected DMAC development. To better demonstrate that DMAC can offer strong fingermark development, the images illuminated with 490 nm are displayed. It is important to note that IND/$ZnCl_2$ does result in the initial Joullié's Pink ridge detail, whereas DMAC treated samples are only very faintly visible as yellow-brown developments under white light.

Both reagents were also used to develop latent fingermarks on thermal paper, where the standard IND/$ZnCl_2$ method was used initially. The low polarity of the DMAC reagent formulation did not cause a discolouration of the substrate, and in combination with the heat-free treatment, yielded clear ridge detail. As expected, IND/$ZnCl_2$ caused a reaction with the thermal paper, where very faint ridge detail could only be observed on some exhibits (Figure 3.5). As the standard IND/$ZnCl_2$ method is not designed for thermal paper (or substrates sensitive to polar solvents), the acid- and heat-free IND/$ZnCl_2$ method was also tested on these substrates [109]. The DMAC method (without applying heat) appeared to be less sensitive and luminescent in this case (mean=1.4, median=1 vs. mean=2.2, median 2). However, the shorter development time makes it much more suitable for routine fingermark treatments than the 2 days required by the acid- and heat-free IND/$ZnCl_2$ method.

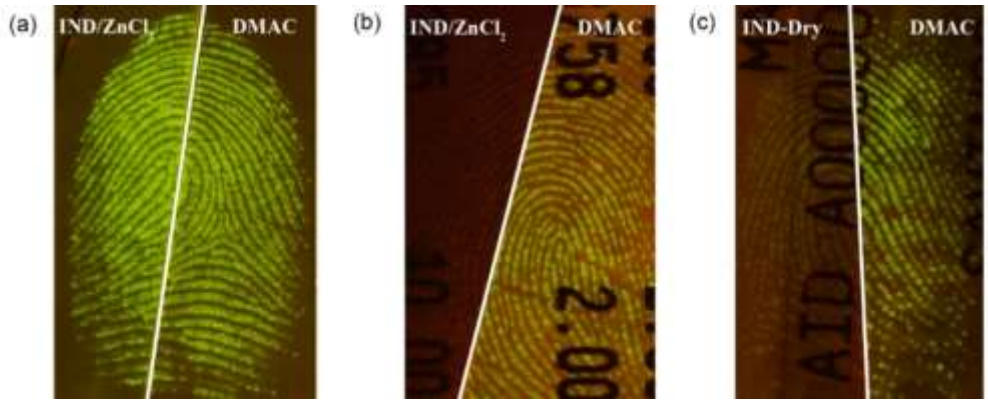


Figure 3.5 (a) IND/$ZnCl_2$ vs. DMAC treated fingermark deposited on white copy paper. (b) IND/$ZnCl_2$ vs. DMAC treated fingermark deposited on thermal paper. (c) IND-dry vs. DMAC treated fingermark deposited on thermal paper. Photographs taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

As in Chapter 2, a blind study can provide a comparison of the performances between two reagents. In this case, ridge detail was observed from all deposited fingermarks following either IND/$ZnCl_2$ or DMAC treatment. A depletion series can give further indication of a reagent's performance, and is of special interest when two methods are compared. It was noted that in this case, insignificant differences were observed between IND/$ZnCl_2$ or DMAC treated depletion series. Interestingly, whilst development was present in nearly all the treated marks, the strength of the ridge contrast was greatly reduced from the second deposit onward for both methods.

As recommended by the IFRG guidelines, DMAC treated fingermarks were compared to an established reagent (in this case IND/$ZnCl_2$) to determine how many exhibits would be suitable for identification purposes [100]. While this is a Phase 1 project, and these recommendations were intended for a Phase 3 study, it was thought that by undertaking this comparison, an early indication of the relative performance could be seen. Unedited images of the treated fingermark exhibits for white copy paper and the depletion series samples were used for the comparison. It was found that both reagents performed the same with regards to identifiable prints, where a 60 % success rate was achieved.

Fingermarks deposited on paper envelopes near the clear window could be developed with both reagents, although IND/$ZnCl_2$ gave better development overall (Figure 3.6). It has to be noted that the Elna laundry press resulted in the melting of the clear plastic window for the IND/$ZnCl_2$ treatment, whereas DMAC was developed by leaving the treated specimen in ambient conditions for 2.5 hours, therefore keeping the clear window intact. The solvents used in either reagent also did not negatively affect the clear window. This test was redone using the acid- and heat-free IND/$ZnCl_2$ approach, where it was again found that DMAC developed the fingermarks to a lesser extent (mean=2.4, median=3 vs. mean=3.4, median 4).

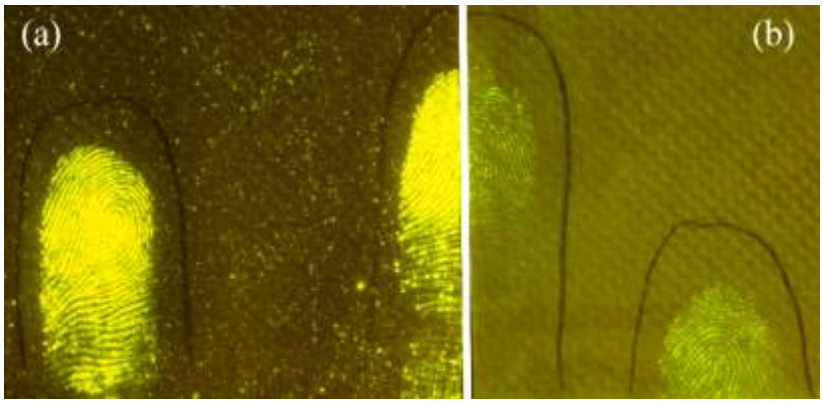


Figure 3.6 Latent fingermarks deposited on a white paper envelope and treated with IND/$ZnCl_2$ (a) or heat-free DMAC (b). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

Post-it notes displayed poor contrast due to intense background colouration and luminescence in the case of IND/$ZnCl_2$ treated fingermarks (mean and median equal to 0). The DMAC developed counterparts exhibited good ridge detail with an acceptable level of background staining, where mean and median grades equal to 3 were found (Figure 3.7). A different brand of this substrate was consequently retested using the acid- and heat-free IND/$ZnCl_2$ approach, where intense background staining occurred with both reagents. Some of the acid- and heat-free IND/$ZnCl_2$ treated fingermarks were of sufficient intensity that ridge detail could be observed despite the poor background contrast.

Figure 3.7 Latent fingermarks deposited on a yellow post-it note and treated with IND/$ZnCl_2$ (a) or heat-free DMAC (b). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

IND/$ZnCl_2$ treated fingermarks on newspaper showed very strong and clear ridge detail, with intense pink colouration (mean=2.6, median=2.5). Due to overdevelopment in some cases, a relatively low score was assigned. In contrast, fingermarks developed using DMAC emitted relatively weak luminescence overall (mean=1.25, median=2.5) (Figure 3.8).

Figure 3.8 Latent fingermarks deposited on newspaper and treated with IND/$ZnCl_2$ (a) or heat-free DMAC (b). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

To ascertain whether the new DMAC formulation still targets the proposed urea content of latent fingermark deposits, a simple urea spot test at 1 g/L and 10 mg/L was treated. Rather surprisingly, no luminescence development was observed at either concentration. These urea spot tests were also treated with IND/$ZnCl_2$, where once again no development was observed. Repeat experiments also did not yield luminescent products with either reagent.

3.3.3 Photoluminescence studies

Solutions of alanine, serine and glycine were used to investigate the luminescence characteristics of the DMAC reaction product. These amino acids were selected due to their reported abundance in latent fingermark deposits [55, 177]. All of the treated high concentration spots appeared as yellow-brown impressions, which exhibited strong photoluminescence when illuminated at 490 or 505 nm and viewed through an orange barrier filter (Figure 3.9). As expected, the colour and luminescence intensity was decreased when viewing the low concentration spots.

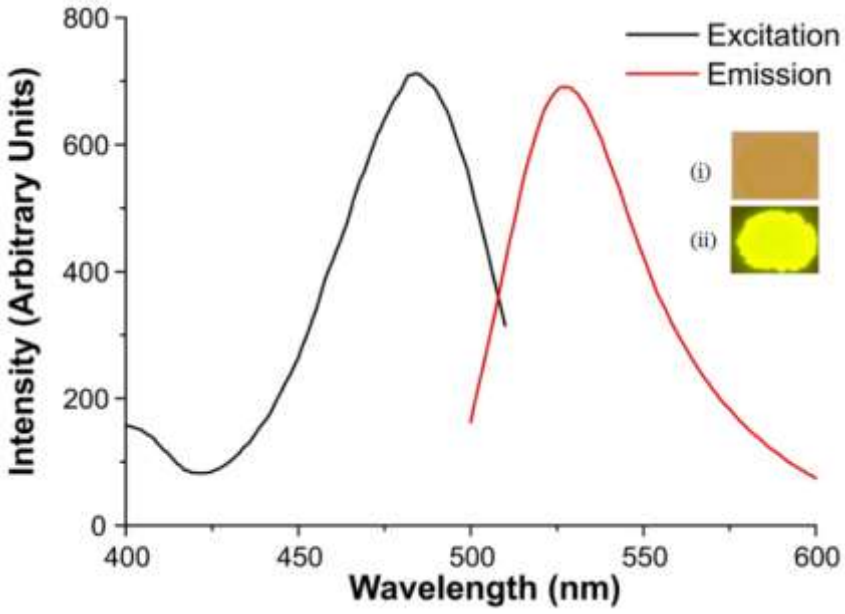


Figure 3.9 Luminescence spectra for a glycine amino acid spot on paper developed with DMAC. Inset image of developed glycine spot on paper. Photograph taken with a Nikon D300 camera, focal length: 60 mm, in (i) absorbance mode; shutter speed: 1/20 second and aperture: f/11; and (ii) luminescence mode at an excitation wavelength of 490 nm; shutter speed: 1 second and aperture: f/11.

Fluorescence spectra of each of the treated amino acids exhibited similar excitation maxima between 480-485 nm. The emission spectra for the treated amino acid spots and latent fingermark were collected using an excitation wavelength of 490 nm (the

nearest available wavelength on the Polilight to the λ_{max} of the glycine reaction product). The emission spectrum for the developed fingermark is very similar to those of the developed amino acids spots (Figure 3.10). The emission and excitation spectra were similar those exhibited by DMAB, as is expected from their structural similarity [167].

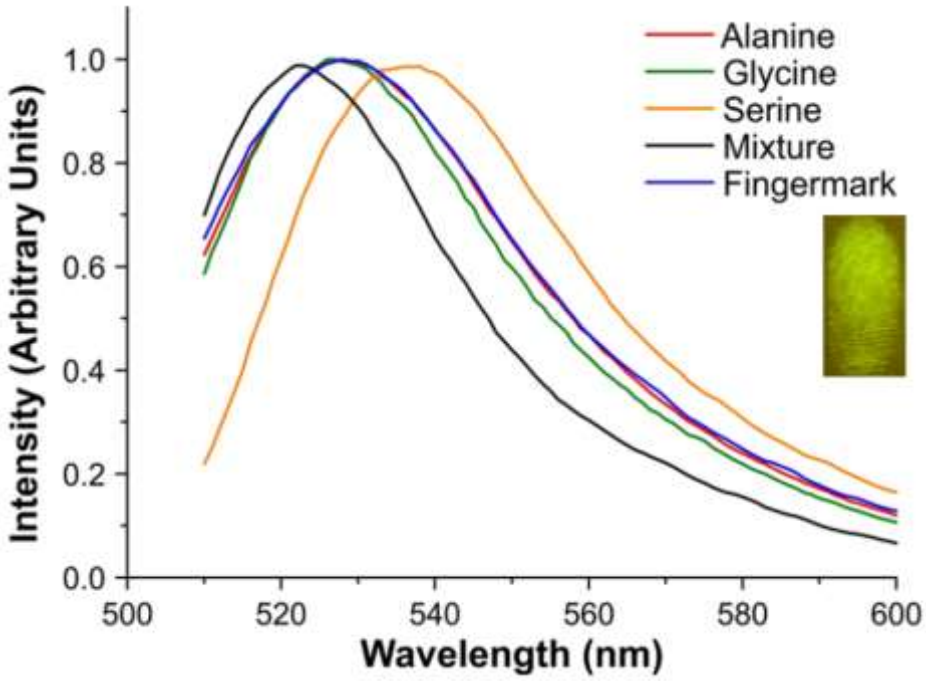


Figure 3.10 Normalised luminescence spectra for amino acid spots (L-alanine, glycine, L-serine and a mixture of the three) and latent fingermark (photograph) on paper developed with DMAC. Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

3.3.4 Interval between deposition and development

Earlier work, such as that of Sasson and Almog, found that DMAC was unable to reliably develop fingermarks older than 72 hours, with the marks being displayed as "unresolved stains" [23]. However, more recent work, such as that by Brennan *et al.* and Ramotowski, indicates that marks older than 4 months or even 12 months could be successfully developed [141, 142]. Fingermark deposits that had been left in ambient conditions for extended periods were treated with the final DMAC working method. Full detail was still observed in fingermarks even after 6 months; however, all samples showed diminished intensity compared to the halves that were developed when fresh. Blurring of the ridgelines, as was the case with the original DMAC method, was not observed (Figure 3.11) [23].

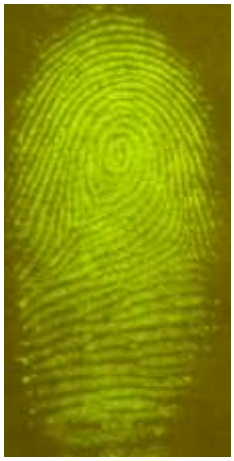


Figure 3.11 6 month old fingermark sample on white copy paper treated with DMAC. Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

3.3.5 Previously published formulations

The wet and dry contact methods used in Ramotowski's study (DMAC USA wet and DMAC USA dry) were applied and compared to the developed DMAC formulation [142]. The DMAC USA wet method stained the exhibit bright yellow and gave very strong background luminescence with no observable ridge detail or initial red colour (Figure 3.12a). It was noted that the DMAC concentration in the DMAC USA wet formulation appeared to be very high and the solution was therefore diluted one hundred-fold in ethanol. This offered very luminescent ridge detail with much lower background fluorescence, and no further background development over time was noted. However, the diluted working solution of DMAC USA wet was not quite as sensitive as the developed DMAC formulation, with the additional disadvantages of solely utilising a polar solvent that causes inks to run, and reacts with the active layer present in receipts consisting of thermal paper (Figure 3.12b). The DMAC USA dry method offered very good results, with little background staining (Figure 3.12c). It was comparable to the developed DMAC formulation on most samples; however, it could not be used for thermal paper due to the required heat application step. The expected formation of a red precipitate was not found to occur with any samples tested in the current study.

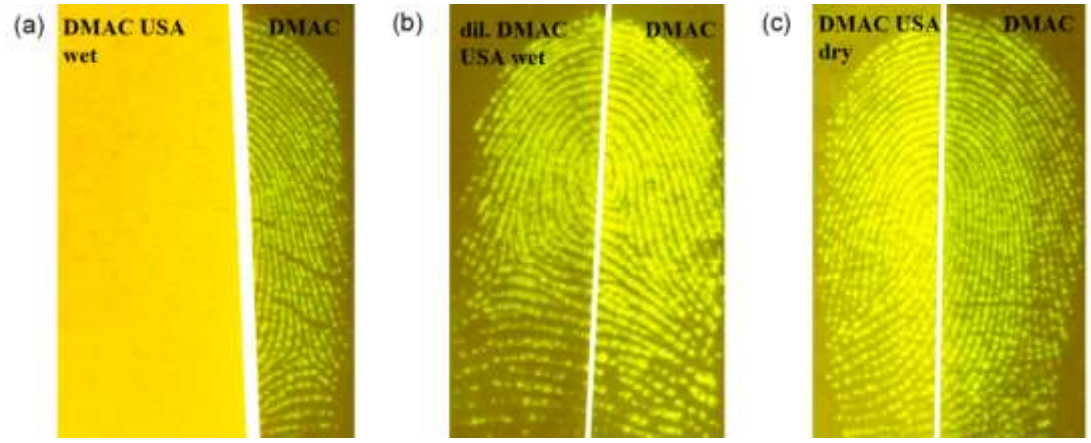


Figure 3.12 (a) Fingermark sample treated with Ramotowski's original wet contact formulation and DMAC. (b) Fingermark sample treated with the diluted formulation of Ramotowski's wet contact reagent and DMAC. (c) Fingermark sample treated Ramotowski's dry contact reagent and DMAC [178]. Substrate: white copy paper. Photographs taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

The dry contact method outlined by Lee *et al.* was found to be an effective fingermark development technique [143]. It offered the same levels of development as the developed DMAC formulation, and may find use for remote deployment as treatment papers can be prepared in advance and kept in sealed zip-lock bags (Figure 3.13). Being a dry contact method, it is also a very gentle approach to examining fragile samples. Lee's dry contact method does however require 24 hours to fully develop ridge detail, which may limit its use in routine police work where a faster turn-around time may be desirable.

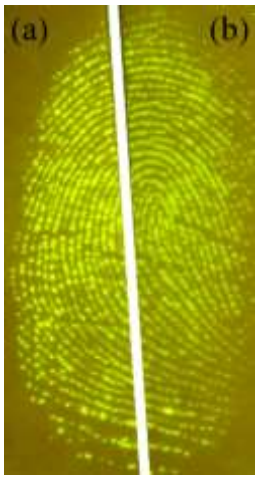


Figure 3.13 Fingermark sample on white copy paper treated by the dry contact reagent proposed by Lee *et al.* (a) and DMAC (b) [143]. Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

3.4 Conclusions

A new and improved formulation of DMAC was established for the detection of latent fingermarks. Good ridge detail was observed following the treatment of porous substrates with DMAC (0.028 g *p*-dimethylaminocinnamaldehyde, 0.84 mL glacial acetic acid, 6.2 mL ethyl acetate and 0.993 L 40-60 °C petroleum spirits). Ethyl acetate and petroleum spirits may be substituted by ethanol and HFE-7100, respectively. The impressions could be visualised after heating at approximately 160 °C for 20 seconds in an Elna laundry press, or by allowing the reaction to go to completion under ambient conditions for 2-3 hours. The low polarity of the solvent used allows the treatment of thermal paper without modification to the formulation. Photoluminescence studies indicate that an excitation wavelength of 480-490 nm yields the highest emission (at ~530 nm). Comparisons to previously published formulations indicate that the new wet contact formulation affords a more rapid and sensitive development of latent fingermarks. Similar development of fingermarks was observed in comparison to those treated with IND/$ZnCl_2$ on all but very weak samples on plain white paper. The number of exhibits suitable for identification purposes was found to be the same for both DMAC and IND/$ZnCl_2$. Based upon these findings, it is recommended that this formulation be more closely investigated in a subsequent Phase 2 study to fully appreciate its development potential.

Further studies are required to more fully investigate the operational potential of DMAB and DMAC for latent fingermark detection. These include studies into a wider range of substrates and the effect of including other components, such as metal salts, in the formulation or as a post treatment. In addition there is a need to synthesise and isolate the photoluminescent imine reaction products. Once the properties of these are better understood there is the potential to rationally design and synthesise analogues of DMAB and/or DMAC that provide improved performance as fingermark detection reagents.

Chapter 4
Variability in the response of 1,2-indanedione/zinc chloride treated latent fingermarks

Portions of this Chapter have been published in the following journal:

P. Fritz, A.A. Frick, W. van Bronswijk, S.W. Lewis, A. Beaudoin, S. Bleay, and C. Lennard, *The variability and subjectivity of fingermark grading – towards a more consistent approach*. Journal Forensic Identification, 2014. **Submitted.**

P. Fritz, W. van Bronswijk, E.L.T. Patton, and S.W. Lewis, *Variability in Visualization of Latent Fingermarks Developed with 1,2-Indanedione–Zinc Chloride.* Journal Forensic Identification, 2013. **63**(6): p. 698-713.

4.1 Introduction

In Chapters 2 and 3, the response of *p*-dimethylaminobenzaldehyde and *p*-dimethylaminocynnamaldehyde treated fingermark deposits were observed to vary depending on the donor. In collaboration with A. Frick (submitted PhD thesis at Curtin University, Western Australia, in January 2015), it was further recognised that while donors could be broadly categorised into strong or weak donor groupings for amino acid sensitive reagents, this did not necessarily correlate to the performance of lipid sensitive techniques. In this thesis, two approaches are used to evaluate the amino acid content of latent fingermark deposits. The first method is to treat exhibits with an amino acid sensitive reagent, and then evaluate the responses of the samples by assigning numerical grades to results. The second approach is to characterise the chemical composition of fingermark deposits using analytical techniques. The first method will be discussed in detail in this chapter, whereas the latter will be disseminated in Chapters 5 and 6.

4.1.1 Donor traits

It has been observed that the quality of 1,2-indanedione/zinc chloride (IND/$ZnCl_2$) treated fingermarks are not uniform within a donor population, where age, sex, prior activity and diet of the donor are thought to be possible causes of the amino acid variation [18, 62, 65, 179, 180]. Although various studies examining the amino acid concentration profile in human sweat have been conducted, these either did not focus on the amino acid variation in latent fingermarks, or had too few donors to be statistically significant [39, 55, 74, 181-184]. The reaction scheme in Figure 1.7 in Chapter 1 outlines the reaction of IND with amino acids. As can be inferred from the mechanism, the type and amount of amino acid present in the fingermark deposits will affect the intensity of the product formed. Similar findings have been seen with amino acid printed spot tests treated with ninhydrin and 1,8-diazafluoren-9-one [148]. This difference in the quality (i.e. amino acid type) and quantity of the reaction products formed upon treating fingermarks may be used to discern variations correlating to the donor.

4.1.2 Grading schemes

In order to determine the potential effect that the donor has on the performance of visualisation methods, a comparative grading scheme has to be applied to the treated samples. Recent guidelines from the International Fingerprint Research Group (IFRG) highlight the requirement for standardised research and validation methods [100]. More specifically, the use of grading scales is highly recommended in order to assess the quality of developed fingermarks for all comparison and validation experiments.

For this purpose, there are several fingermark grading schemes currently in use, tailored to suit specific investigations [100, 145, 174, 185-191]. For example, the grading scale proposed by McLaren *et al.* is widely used to compare the performance of two different treatment methods, by using a range from -2 (less effective) to +2 (more effective) to compare a new (or dependent) method to the known (or independent) method [188]. Another commonly employed technique, also used in this Chapter, is that devised by the Home Office Police Scientific Development Branch (HOPSDB), UK (Table 2.3 in section 2.2.16) [174]. This uses a 5-point system grading fingermarks from 0 (no development) to 4 (full development with continuous ridges and excellent contrast), giving absolute values for each individual sample.

The main issue with such ranking systems is their subjectivity; assessment of fingermark quality can only be carried out by human observation and evaluation, and as such is subject to bias stemming from an individual's experience and personal idea of what constitutes 'good' fingermark development [145, 191]. It is known that these differences in personal opinion cannot be completely controlled by assessment protocols, with similar issues having been noted in the fingermark identification process [192].

4.1.3 Aims

A pilot project was first undertaken to see whether any differences between fingermark deposits collected from a wide age range and both sexes can be found. This study will indicate whether the collection of large sample sets is feasible, how well the IND/$ZnCl_2$ reagent performs across a varied donor population and whether the 5-point grading system is adequate as a ranking scale. This trial also investigated if any correlations exist between the grades and the individual donor traits (e.g. age and sex), and which statistical methods are appropriate to use for data interpretation.

Depending on the outcomes of the pilot study, the variation in fingermark assessment by researchers from different institutions, geographical locations, and varying familiarity with latent fingermarks would be assessed. This may address some of the concerns and uncertainty that are presently associated with the subjectivity of fingermark evaluation. Based on the success of the initial work, fingermarks were collected from a larger population of donors, treated with IND/$ZnCl_2$ and ranked according to the quality of the mark by a number of graders. Statistical means were then employed to determine any significance that might arise due to the age of the sample or donor traits.

4.2 Materials and method

4.2.1 Collection and treatment of latent fingermarks

All fingermark donors were required to sign a Human Resources Ethics Committee approval form prior to the collection of any samples (Approval Number SMEC-94-11). If donors were under the age of 18 at the time of collection, signatures from a parent or guardian were required. Further information, such as the donor's age, biological sex, activity and recent habits was also collected; see Appendix A for a sample questionnaire. The conditions were kept as natural as possible with no 'charging' of fingermarks (rubbing of fingers on sebum rich body parts) prior to deposition. Donors were instructed to gently place uncharged index, middle and ring fingertips onto white copy paper (Fuji Xerox Professional, 80 g/m^2) for 10 seconds, where the middle finger was placed on the printed line separating two squares (3-print as outlined in Figure 2.3 in Chapter 2). Samples were treated within 36 hours of

deposition unless otherwise stated. All collected samples were treated with the IND/$ZnCl_2$ method outlined in Chapter 2. Samples for the pilot study were collected and treated by E.L.T. Patton as part of the BioGenius Challenge of Western Australia in 2009.

4.2.1.1 Pilot study

Details of the 120 donors from which latent fingermarks were collected are given in Table 4.1.

Table 4.1 Donor information with regards to the number of donors for each variable (n=120).

Variable	Grouping	Number of Donors
Biological sex	Male	*60*
	Female	*60*
Age	Over 25	*69 (M:37, F:32)*
	Under 25	*51 (M:23, F:28)*
Food Consumption	Yes	*28*
	No	*92*
Washing of hands	Yes	*30*
	No	*90*

4.2.1.2 Grading study

Latent fingermarks were collected from four donors. Donors had not consumed food or handled chemicals for at least 30 minutes before providing samples. Prior to fingermark collection, a laser printer was used to print 20 squares on each page which were cut out after sample collection. A depletion series was collected from both hands, where donors deposited a print in each square giving a total of 20 samples per donor.

4.2.1.3 Donor study

Fingermark samples were collected over a period of 18 months from locations comprising the Curtin University Bentley campus, primary schools, retirement villages and special interest groups located in Perth, Western Australia. Fingermarks were collected on white copy paper with printed gridlines. These gridlines formed 16 squares and facilitated the collection of a 3-print depletion series of 4 impressions

with each hand, where the 3-prints were split as illustrated in Figure 2.3 in Chapter 2. Fingermark samples from 131 donors were collected, with the age and biological sex distribution, as well as additional information, presented in Table 4.2. One half of the split samples were treated with IND/$ZnCl_2$ within 3 days, and the other half 1 month later (stored in a darkened cupboard in controlled laboratory conditions).

Table 4.2 Donor information with regards to the number of donors for each variable in the donor study (n=131).

Variable	Grouping	Number of Donors
Biological sex	Male	*67*
	Female	*64*
Age	30 and over	*56 (M:31, F:25)*
	Under 30	*75 (M:36, F:39)*
Food Consumption	Yes	*69*
	No	*62*
Washing of hands	Yes	*57*
	No	*74*
Recent cosmetics (< 12 hrs)	Yes	*52*
	No	*79*

4.2.2 Data distribution and assessment of developed latent fingermarks

4.2.2.1 Pilot study

All fingermark images were graded by the same person for the pilot study. The results were recorded in Microsoft Excel Professional Plus 2010. All treated fingermarks in this Chapter were graded using a 5-point system based on that used by the HOPSDB (Chapter 2, Table 2.3) [174].

4.2.2.2 Grading study

For the grading study, 80 original and 20 duplicated (randomly selected) fingermark images were ranked by 11 graders. To reduce the effects of exhaustion, stress, etc., and to make the process less overwhelming, the samples were distributed to the graders in 5 batches. This was implemented by numbering the samples and then randomly assigning them to one of the batches using the random number generator in Microsoft Excel Professional Plus 2010. The images were distributed to fingermark assessors via an online cloud program, Dropbox (v.1.4.8). Each assessor was

required to grade their batch of samples using the same scale as for the pilot study, before the next batch of images was released.

4.2.2.3 Donor study

For the donor study, 262 original sample images and 19 replicates were ranked by 5 graders. As per the grading study, batches of sample images were distributed to the graders. For the sample randomisation and distribution process, refer to the grading study above.

4.2.3 Statistical analyses

Distribution-free, or non-parametric, tests do not require the assumption of normally distributed data and compare the medians rather than the means [193]. The equivalent of the parametric independent t-test is a Mann-Whitney U test, assessing the probability that the medians are significantly different [194]. The equivalent of a paired t-test is the Wilcoxon signed rank test, which was used on all paired data, such as ageing of the samples, duplicate grades, etc. [195]. Inter-grader consistency was assessed using intraclass correlation coefficients [196, 197]. Independent data was evaluated using the Mann-Whitney U test. The calculated z value is compared to the critical value, which is ±1.96 at the 95 % confidence level, where $z_{calc} > z_{critical}$ indicates that the difference is significant [198]. The calculated probability values (p values) below 0.05 indicate that the null hypothesis (i.e. that no significant difference exists) can be rejected with greater than 95 % confidence. The Mann-Whitney U test, intraclass correlation coefficients and Wilcoxon signed rank test analyses were performed with IBM's SPSS Version 2.0.

4.3 Results and discussion

4.3.1 Pilot study

It can be difficult to observe subtle changes when viewing IND/$ZnCl_2$ treated fingermarks in photoluminescence mode due to their very strong luminescence. To make the grading process simpler, all luminescent photos where transformed to negative black and white images. The grades were recorded from the resulting images. A typical example of a freshly developed sample and its state after 3 years ageing is shown in Figure 4.1.

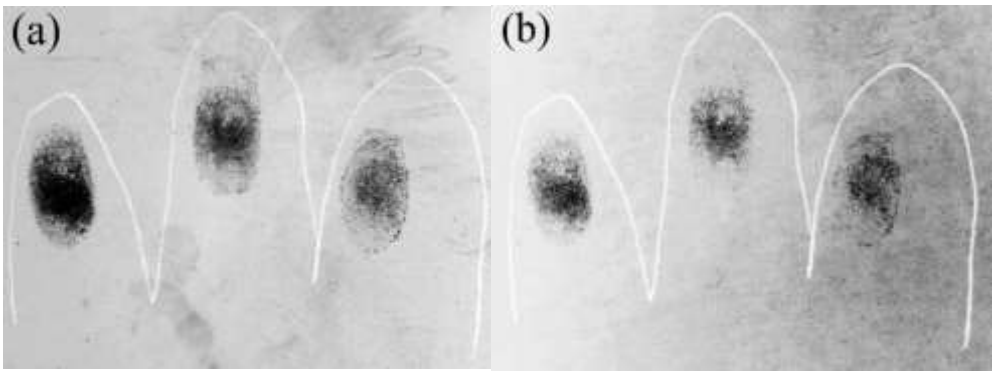


Figure 4.1 Photographs (negative) of a luminescent fresh fingermark sample (a) and after 3 years (b). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

As the prime interest of this study lay with the variation with which an amino acid sensitive reagent treats latent fingermarks (and therefore the relative amounts of amino acid in sweat within a population), the data was evaluated using statistical analyses, rather than reviewing individual results. While this study was commenced as a school project, the wealth of information that could be gained by further analyses of the results quickly became apparent. To this effect, the Mann-Whitney U test and the Wilcoxon signed rank test were employed to investigate the variation in fingermark grades and therefore the relative amounts of amino acids present within a population.

4.3.1.1 Ageing of developed fingermarks

As depicted in Table 4.3, the Wilcoxon signed rank test indicates that the grade obtained from freshly deposited and treated fingermarks (median ($\mu_{1/2}$) = 3, standard deviation (SD) = 1.12) are statistically dissimilar to the grades obtained 3 years later

($\mu_{1/2}$ = 2, SD = 1.19). The Z score of -8.35 is much greater than the $z_{critical}$ (-1.96) and the probability of incorrectly having rejected the null hypothesis (p) is 1.3×10^{-7}. The $\mu_{1/2}$ value shows the median grade that was achieved for that variable, with the SD indicating how close the spread of all the grades are to this median value (lower SD indicates that the grades lie closer together). The large Z score and low p value attained in the Wilcoxon signed rank test indicate that there is a substantial and significant statistical difference between the medians of the grades attained.

Table 4.3 Statistical values calculated using the Wilcoxon signed rank test.

Age of sample	Fresh	Aged
Number of Donors	120	120
Median	3	2
Mean	2.74	1.89
Standard Deviation	1.12	1.19
p-value	1.3×10^{-7}	
Z score	-8.35	

Although non-parametric tests do not require the calculation of means and standard deviations, they have been included to give a better indication of the distribution of the grades than the integers of the medians can give. In addition to the results from the statistical techniques, the number of donors contributing to a fingermark grade of zero increased from 0 in 2009 to 13 in 2012. Also, 40 samples achieved the highest grade of 4 in 2009, which decreased to 12 in 2012 (Table 4.4). These results suggest that degradation of the Joullié's Pink complex is occurring as a function time. Previous research also suggests that treated samples degrade over time, especially when the reagent formulation does not include zinc [113, 120]. However, the extent of the decomposition is not described in these studies.

Table 4.4 The distribution of the fingermark grades in samples from 2009 and 2012, in reference to the number of donors.

Grade	Number of Donors, 2009	Number of Donors, 2012
0	0	13
1	23	40
2	25	26
3	32	29
4	40	12

4.3.1.2 Donor variability

As previously stated, the quality of IND/$ZnCl_2$ treated fingermarks is not uniform within a donor population, with age, sex, prior activity and diet thought to be possible causes of amino acid variation [18, 65, 179, 180, 183]. Figure 4.2 is an example where the difference is easily visualised; however, in Figure 4.3 the differences are much more difficult to observe. In order to deduce meaningful information from the data, rigorous statistical analyses have to be applied.

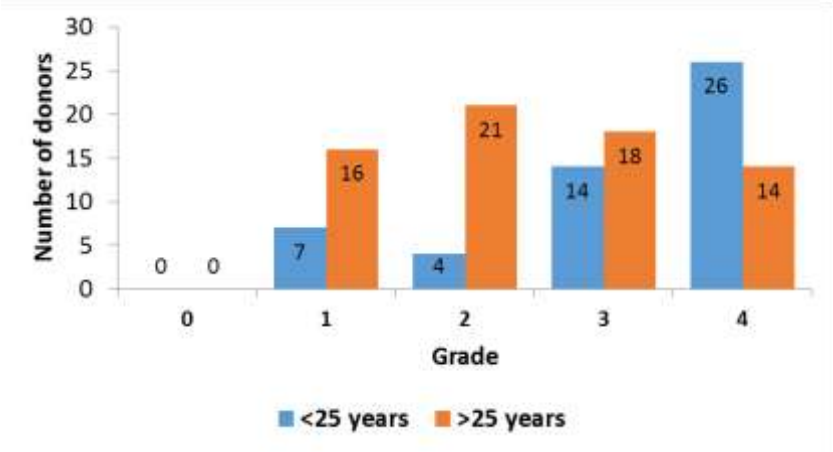


Figure 4.2 Variability in quality for IND/$ZnCl_2$ developed fingermarks on paper as a function of donor age.

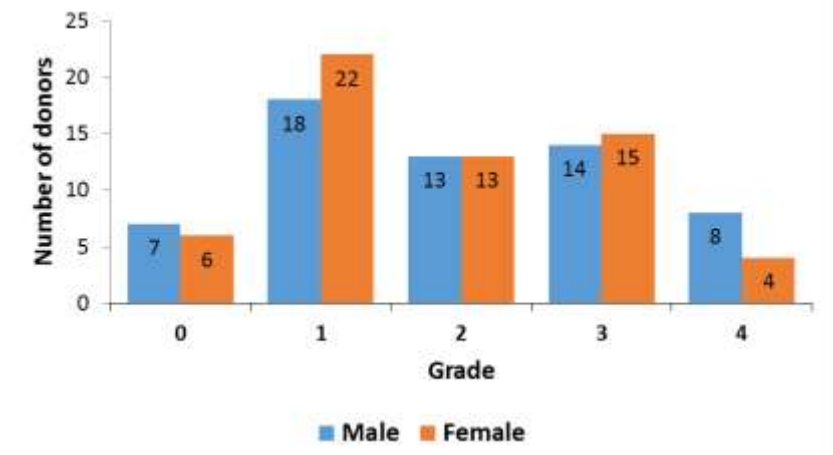


Figure 4.3 Variability in quality for IND/ZnCl2 developed fingermarks on paper as a function of sex.

To this effect, the non-parametric Mann-Whitney U test was used to evaluate if statistically significant disparity exists between the non-paired variables of sex, donor age, food consumption and washing of hands (Tables 4.5 and 4.6).

Table 4.5 Statistical values gained from Mann-Whitney U tests, with the fresh luminescent fingermark grade given as a function of the independent variables.

Variable	Donor age		Washing of hands		Biological sex		Food consumption	
	Over 25	Under 25	Yes	No	Male	Female	Yes	No
# of Donors	69	51	30	90	60	60	28	92
Median	2	4	2	3	3	3	3	3
Mean	2.44	3.16	2.20	2.92	2.67	2.82	2.61	2.78
Standard Deviation	1.09	1.19	1.01	1.21	1.25	1.13	1.07	1.20
U score	1101		880.5		1741.5		1166	
Z score	-3.63		-2.95		-0.32		-0.79	
p-value	2.9×10^{-4}		3.2×10^{-3}		0.75		0.43	

The initial (2009, fresh) grades from donors over ($\mu_{1/2}$ = 2, SD = 1.09) or under the age of 25 ($\mu_{1/2}$ = 4, SD = 1.07) were found to be significantly different ($p = 2.9 \times 10^{-4}$, Z = -3.63) (Table 4.5). The Z score is -3.63, which greatly exceeds the critical *z* value of -1.96 and the null hypothesis can be rejected with greater than 99.97% confidence. In effect, the statistics support the idea that there are significant differences between donors over or under the age of 25. In this study, 34 out of the 51 donors under the age of 25 were aged 15 years or younger. Previous research suggests that there is a marked difference, especially within the lipid fraction, between the chemical profile of children's and adults' latent fingermarks [9, 65, 179, 180, 199]. Buchanan *et al.* explained that surface lipids in children occur due to the epidermis (outer skin layer), rather than sebaceous glands in adults, with sebaceous secretions increasing following puberty [28]. Further, Williams *et al.* found that the main difference between adults' and children's deposits was the relative ratios of compounds and that proteins, themselves comprised of various amino acids, make up less than 1 % in children, but around 5 % in adults [199].

A similar statistically significant disparity was observed in the initial fingermark grades as a function of the donors washing their hands ($\mu_{1/2}$ = 2, SD = 1.16) or not prior to fingermark deposition ($\mu_{1/2}$ = 3, SD = 1.09, $p = 3.2 \times 10^{-3}$, Z = -2.95) (Table 4.5). Again the very low p-value and the large Z score indicate with greater than 99.6% confidence that the medians of this variable are different. This is to be expected, as more of the water-soluble amino acids are present on the surface of unwashed hands and are thus transferred and available to react with IND/$ZnCl_2$.

There appears to be no statistically significant dissimilarity between donors who had recently consumed food or not ($p = 0.43$). The sex of the donor ($p = 0.75$) was also not found to have a significant effect on the fresh fingermark grade (Table 4.5), i.e. there did not appear to be a contribution to the fingermark grade from either food residues or the sex of the donor. With regards to the food consumption, this only refers to donors that physically handled food with their hands and not the effect their diet has on fingermark deposits. Although there is speculation that sex may affect the chemical composition of fingermark deposits, no conclusive results have been found in the literature and these Mann-Whitney test outcomes are not surprising.

All binary combinations of the variables were also investigated, by comparing sub-groups (age and sex, e.g. female donors over 25 and under 25, etc.). No significant interactions were found, with the exception of the washing of hands, which was found to override the age of donor factor. Washing would remove appreciable quantities of amino acid and hence diminish the age-related difference. This implies that the washing of hands is more crucial to the fingermark grade and overrides the observed age differential.

All of the trends found for the initial grades above were also observed in the grades from independent variables for 3 year old (2012) fingermarks (Table 4.6). However, in the case of food consumption, the aged prints returned a higher grade if the donors had not consumed food, as opposed to those who had eaten recently. This would suggest that chemical components transferred onto the hands from the food increases the rate of degradation for the Joullié's Pink complex.

Table 4.6 Statistical values calculated from Mann-Whitney U tests, with the aged luminescent fingermark grade given as a function of the independent variables.

Variable	**Donor age**		**Washing of hands**		**Biological sex**		**Food consumption**	
	Over 25	**Under 25**	**Yes**	**No**	**Male**	**Female**	**Yes**	**No**
# of Donors	69	51	30	90	60	60	28	92
Median	1	3	1	2	2	2	1	2
Mean	1.48	2.45	1.43	2.04	1.97	1.82	1.54	2.00
Standard Deviation	1.01	1.19	1.01	1.21	1.25	1.13	1.07	1.20
U score	959.5		968.5		1681.5		994	
Z score	-4.39		-2.39		-0.64		-1.89	
p-value	1.2×10^{-5}		1.7×10^{-2}		0.52		0.06	

Upon completion of this preliminary study, it was noted that some of the images were difficult to classify to a particular grade. In addition, the consistency of this grading scheme had thus far not been established. Although only one person had graded all fingermark samples in order to minimise potential intra-grader variation, it was also not known whether the selected grading system could be reliably replicated for the same grader. As such, a new investigation was commenced in collaboration with A. Frick to assess these uncertainties. The findings of that study would then determine whether this preliminary pilot investigation is statistically valid, and whether a larger repeat study would be appropriate.

4.3.2 Grading study

The grading study was undertaken to evaluate whether intra- and inter-grader variation exists when applying grading schemes to the classification of latent fingermark development. 11 fingermark graders were asked to evaluate the same 100 fingermark samples, giving an indication of the robustness of the method, and whether their disparate professional backgrounds and expertise had any impact on the assessments. It is important to note that the ability to examine treated fingermarks for identification purposes was not investigated, but rather the assessment of fingermark development quality. An absolute scale, adapted from Bandey [174], has been used in Chapters 2 and 3 to assess reagent performance. This requires an individual to take into account contrast, clarity and ridge continuity. For the purpose of this study, detailed descriptions of each grade with examples were provided to the graders to reduce bias and encourage more consistent results (see section 2.2.16).

4.3.2.1 Intra-grader variation

It is imperative that an individual grader can perform consistently. If individual graders are unable to dependably assign a grade to identical fingermark images, then no meaningful conclusions can be drawn from any study involving subjective evaluation methods. To investigate this effect, the grades given to the 20 replicate images were examined for each fingermark grader. It was found that 172, or 78.2 %, of the replicate grades were identical to their original scores (Figure 4.4). 48 (21.8 %)

of the replicates showed a difference of 1 grade between the samples, and none of the replicate samples showed a difference of 2 or more grades.

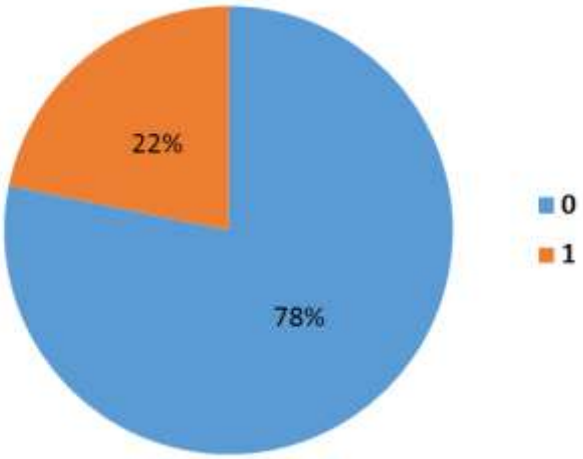


Figure 4.4 Differences between two grades assigned to replicated images.

Half of the replicate images were assigned differing grades by at least two graders, indicating that these may be samples that are borderline (in between two categories) or otherwise difficult to categorise. Overall, it was found that very low and very high fingermark grades were the most consistently assigned. The majority of disagreements were found to occur with images that were assigned a 2 or 3 rating in at least one instance. These samples, for example, may have exhibited good contrast or detail but also had smudged portions, or did not show continuous ridgelines (Figure 4.5). The more ambiguous nature of these samples in terms of their quality may cause graders to rely more on their own individual idea of a fingermark grade rather than adhering to the grading scale provided.

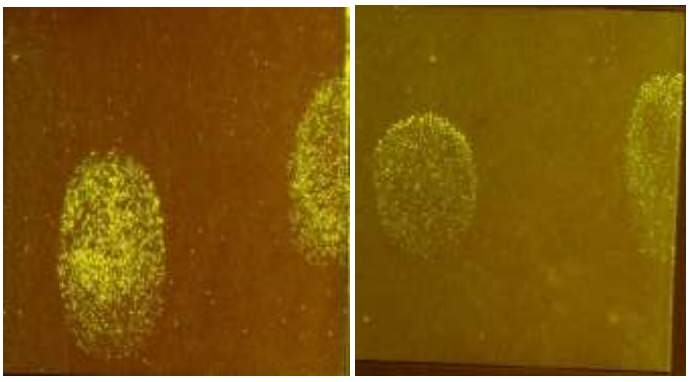


Figure 4.5 Examples of replicated fingermark images graded inconsistently (left) and consistently (right) by the same individual. Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

Wilcoxon signed rank tests were performed on the grades assigned by each grader to the duplicated image pairs (Table 4.7). It was found that no significant difference existed between the grades of the original and duplicated samples for any grader (mean Z = -0.702, mean p = 0.53). The relative experience of each grader with latent fingermarks appeared to have no significant impact on each individual's ability to grade fingermarks consistently.

Table 4.7 Statistical values gained from the Wilcoxon signed rank tests, where the original scores given by each grader were compared to the duplicates ones (n=20).

Grader	1	2	3	4	5	6	7	8	9	10	11
Median (original)	1	1	2	3	2	2	1	2	2	1	2
Mean (original)	2.15	2.1	2.35	3	2.3	2.4	2.1	2.3	2.25	1.95	2.4
Median (duplicates)	1	1	2.5	3	2	2	2	2	2	2	2.5
Mean (duplicates)	2.2	2	2.4	3	2.4	2.4	2.3	2.35	2.3	2.05	2.5
Std. Dev. (original)	1.39	1.33	1.14	0.73	1.08	1.11	1.33	1.08	1.16	1.19	1.31
Std. Dev. (duplicates)	1.44	1.26	1.11	0.92	1.11	1.11	1.38	1.09	1.26	1.05	1.24
p-value	0.56	0.16	0.66	1.00	0.32	1.00	0.10	0.56	0.66	0.41	0.41
Z score	-0.58	-1.41	-0.45	0.00	-1.00	0.00	-1.63	-0.58	-0.45	-0.82	-0.82

4.3.2.2 Inter-grader variation

Having established that the 11 graders performed consistently within the circumstances of this investigation, comparisons between grader performances were conducted to examine any general trends. Figure 4.6 gives an overview of the absolute distribution of grades assigned for the 100 fingermark images by the 11 graders. For 42 samples, the grades given differed by one amongst the graders, and for a further 53 samples, there was a difference of two grades. For 3 images, there was unanimous agreement on a grade between all graders. The remaining two images were graded the most inconsistently between the 11 graders, with a total difference of 3 grades assigned to these images. Agreement between all graders was most frequent when assessing fingermarks that exhibited very strong or very weak development (Figure 4.7). At first glance, the frequent disagreements between graders appeared to indicate that the grading scale does not appear to be a reliable indication of fingermark development quality. However, this data only accounts for the absolute distribution of grades, rather than any consensus reached between the graders.

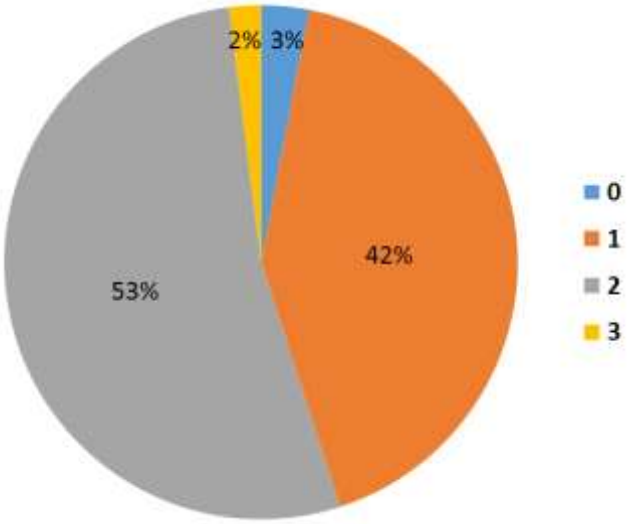


Figure 4.6 Absolute distribution of grades assigned to the 100 treated fingermark samples.

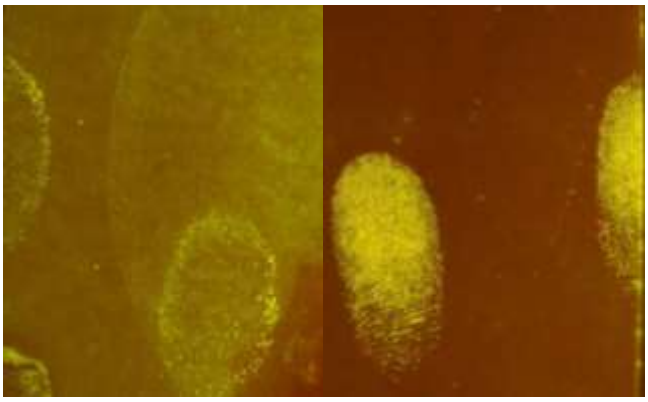


Figure 4.7 Examples of fingermark images unanimously assigned a grade of 1 (left) and 4 (right). Photograph taken with a Nikon D300 camera in luminescence mode at an excitation wavelength of 490 nm; focal length: 60 mm, shutter speed: 1 second and aperture: f/11.

When grades were compared to the median grade for each image, there was better agreement between all 11 graders (Figure 4.8). Total agreement between the assessors' grade and the median occurred in 66.9 % of the 1100 grades assigned in total, and a difference of 1 between the grade and the median occurred in 32.0 % of cases. Therefore, 98.9 % of all grades provided only differed by one or less from the median score assigned to each sample. The remaining 1.1 % of grades differed by 2 from the median. No instance occurred where there was a disagreement of 3 or 4. In light of these statistics, the grading scale appears to be much more robust.

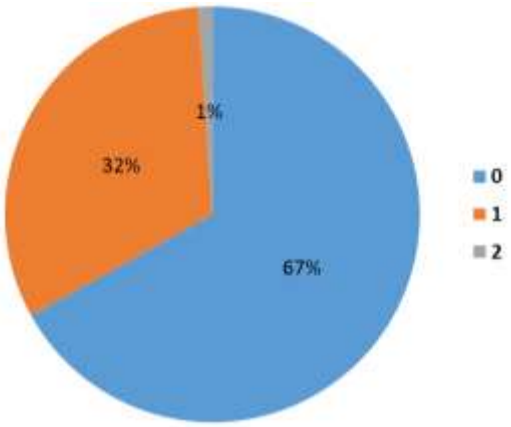


Figure 4.8 Differences between grades given to fingermarks and the median grade for each sample.

While there was general agreement between all graders and the median, there were significant differences in how individual graders performed regarding how frequently they agreed with the median. In some cases, graders agreed with over 85 % of the median grades for each fingermark image, while other graders only agreed with 40 – 50 %. Obviously, this has a large impact on the above results as considerably more samples would be in agreement, as a percentage, without these graders. This is also reflected in the mean of each individual sample rather than the median. As the median reflects the grade most commonly given for each exhibit, it is less affected by individual outliers. The mean, however, does display the skewing of these samples due to differences in grading. On the whole, there was strong agreement between the median and the mean values, where skewing indicated that there was no trend of either over- or under-scoring. It is interesting to note that, while these assessors may not agree with the median grades as strongly as other examiners, their grading consistency was no different.

Furthermore, the performance of each grader appeared to have no correlation to the institution or geographical location; however, graders with greater experience in latent fingermark research tended to give grades that disagreed with the median more often than the more inexperienced graders. It may be that with greater experience, these graders have formed their own standards for fingermark quality, and that these opinions subconsciously influenced their performance even while using the provided grading scale. Conversely, the less experienced graders, some of whom were completely unfamiliar with fingermarks, were more likely to rely almost solely on the grading scale as a guide [192, 200].

The inter-grader variation was also evaluated using intraclass correlation coefficients, which establish the consistency between two or more measurements [197, 201]. The intraclass correlation coefficient was given as 0.973, indicating very strong agreement between all 11 graders. The lower and upper confidence intervals show that 95 % of the time, the assigned grade will give a correlation between 0.964 and 0.981.

Overall, use of this grading scale appears to be a method that offers consistent and robust results for the assessment of fingermark samples and is therefore seen as a feasible approach for use in a pending large-scale donor study. Importantly, these findings further support that the findings of the preliminary pilot study were based on a grading scale that has now been shown to be reproducible and consistent.

4.3.3 Donor study

Fingermark samples from 131 donors from a range of ages of both biological sexes were collected in a collaborative study with A. Frick (Table 4.2). In a similar manner to the pilot study, one half of the split fingermark samples were treated with IND/$ZnCl_2$ within 3 days of their collection. However, unlike the pilot study where all samples were regraded 2 years after their treatment, the second half of the donor study samples were treated and subsequently graded one month after the first side. The median grade of 5 fingermark graders was used for all statistical purposes, including the Wilcoxon signed rank test and Mann-Whitney U tests. Repetition of the statistical tests using the average scores of the 5 graders showed no difference to the results using median values. This study highlighted the efficacy of IND/$ZnCl_2$, as only 6 samples out of a total 1310 grades given (i.e. 0.5 %) returned a score of 0 and 64.6 % of all grades given were a 3 or 4.

4.3.3.1 Grader variation

As a form of quality control, 19 fingermark images were replicated and graded at different points in time by all graders. 78 % of the duplicated images were graded the same as the originals, which is similar to the grading study result (Figure 4.9). As before, the intra-donor variation was observed more often in fingermark samples that may be hard to classify (i.e. grades of 2 or 3).

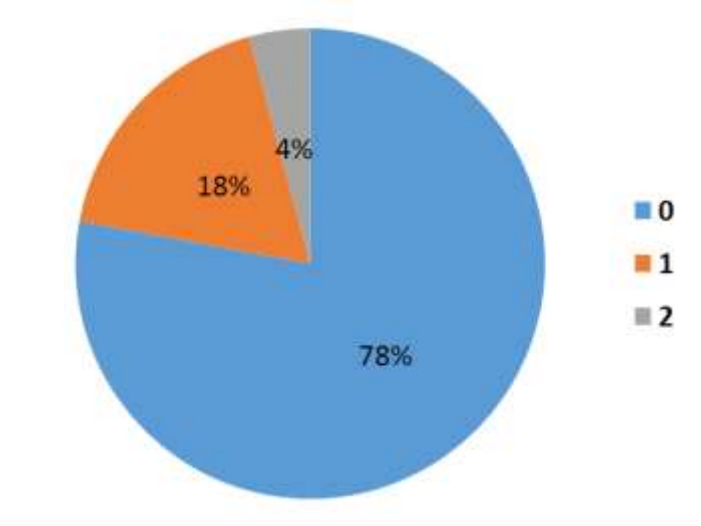


Figure 4.9 Differences between grades assigned to replicated images.

Wilcoxon signed rank tests were performed (Table 4.8), where it was found that no significant difference exists between the original and duplicated grades for any grader ($Z = -0.927$, $p = 0.38$). The grades given by grader 1 were consistently lower than those of the other graders, where it is thought that this is due this grader's much more expansive experience with fingerprints. However, the intraclass correlation coefficient value of 0.847 demonstrated that there was still a strong agreement between all 5 graders. The lower and upper confidence intervals show that 95 % of the time, the assigned grade will give a correlation between 0.749 and 0.899.

Table 4.8 Statistical values gained from the Wilcoxon signed rank tests, where the original scores given by each grader were compared to the duplicates ones (n=19).

Grader	1	2	3	4	5
Median (original)	2	3	3	3	3
Mean (original)	2.1	2.6	2.9	2.4	3.1
Median (duplicates)	2	3	3	3	3
Mean (duplicates)	2.2	2.8	2.8	2.5	3.3
Std. Dev. (original)	1.10	1.16	0.94	1.22	0.94
Std. Dev. (duplicates)	1.01	0.92	1.01	1.31	0.82
p-value	0.56	0.56	0.16	0.38	0.23
Z score	-0.58	-0.58	-1.41	-0.88	-1.19

4.3.3.2 Ageing of deposited fingermarks

Having established that the fingermarks were graded consistently, statistical approaches could be applied to the sample data. In the pilot study, the relative stability of the IND/$ZnCl_2$-amino acid reaction product was evaluated. In this project, the effect that natural deposit ageing may have on fingermark grades was compared to fresh fingermarks (Table 4.9).

Table 4.9 Statistical values calculated using the Wilcoxon signed rank test on the median results of 5 graders.

Age of sample	Fresh	1 month old
Number of Donors	131	131
Median	3	3
Mean	2.86	2.97
Standard Deviation	0.95	0.86
p-value	0.047	
Z score	-1.984	

The results of the Wilcoxon signed rank test indicate that the median grades of treated fresh fingermarks ($\mu_{1/2}$ = 3, SD = 0.95) are not statistically similar to the median grades of sample halves treated 1 month later ($\mu_{1/2}$ = 3, SD = 0.86). The Z score of -1.98 is greater than the $z_{critical}$ (-1.96) and the probability of incorrectly having rejected the null hypothesis (p) is 0.047. The closeness of both the Z score and the p value to the decision making values indicates that while the null hypothesis is rejected, there may not be a strong statistical difference between the medians of the grades attained. This can be seen in Table 4.10 and Figure 4.10, where the very similar distribution of grades given for the fingermark samples may suggest that the effect of ageing (at least within this timeframe) is minimal.

Table 4.10 The distribution (%) of grades given for fingermarks treated immediately and after one month.

Grade	Fresh (%)	1 month old (%)
0	1	0
1	15	14
2	21	20
3	30	33
4	33	34

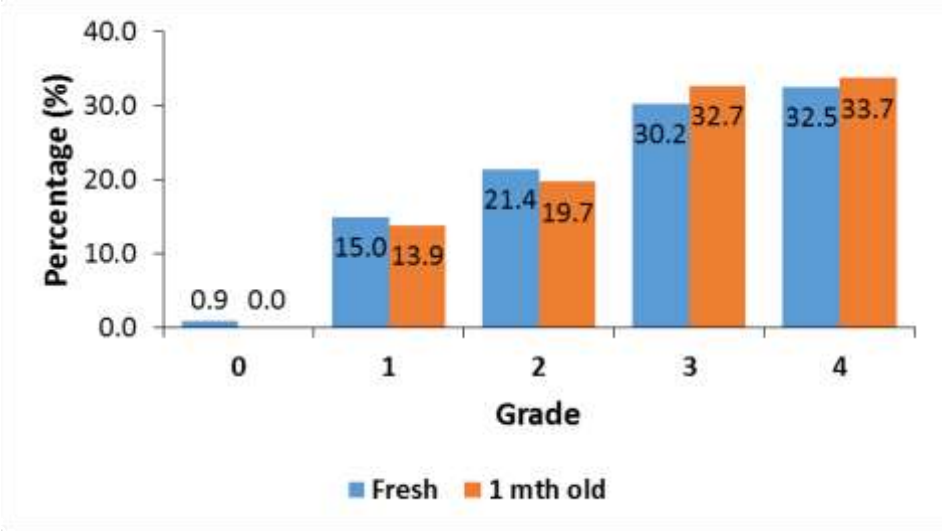


Figure 4.10 Variability in quality for IND/$ZnCl_2$ developed fingermarks on paper as a function of the age of the sample.

4.3.3.3 Donor variability

The treatment of fingermark deposits of 131 further donors may reinforce the findings of the pilot study. As per the findings discussed in section 4.3.1, grades given to some donor traits could be readily distinguished, for example the effect of the age of the donor (Figure 4.11). As Figure 4.12 demonstrates with the biological sex of the donor, other traits may appear to offer negligible differences. Therefore, the non-parametric Mann-Whitney U test was again used to discern whether there were statistical differences between the independent donor traits.

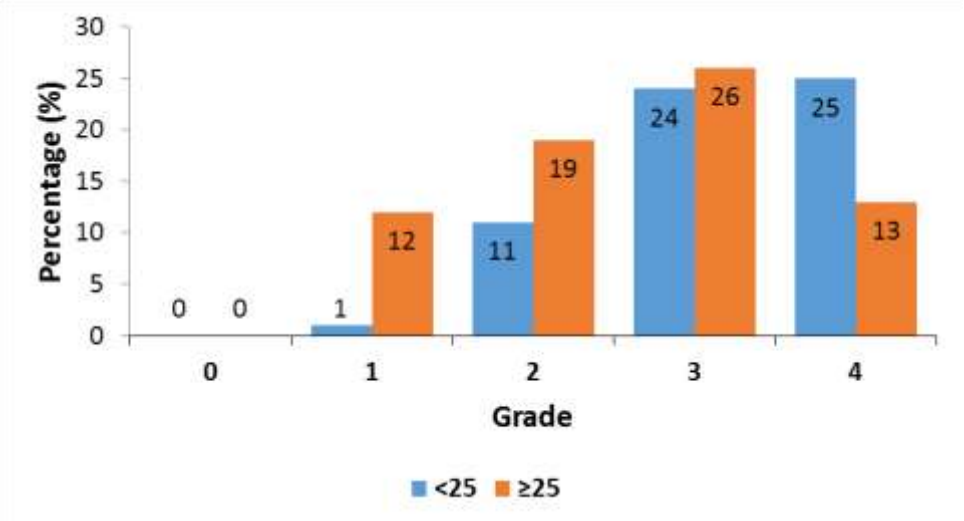


Figure 4.11 Variability in quality for IND/$ZnCl_2$ developed fingermarks on paper as a function of donor age.

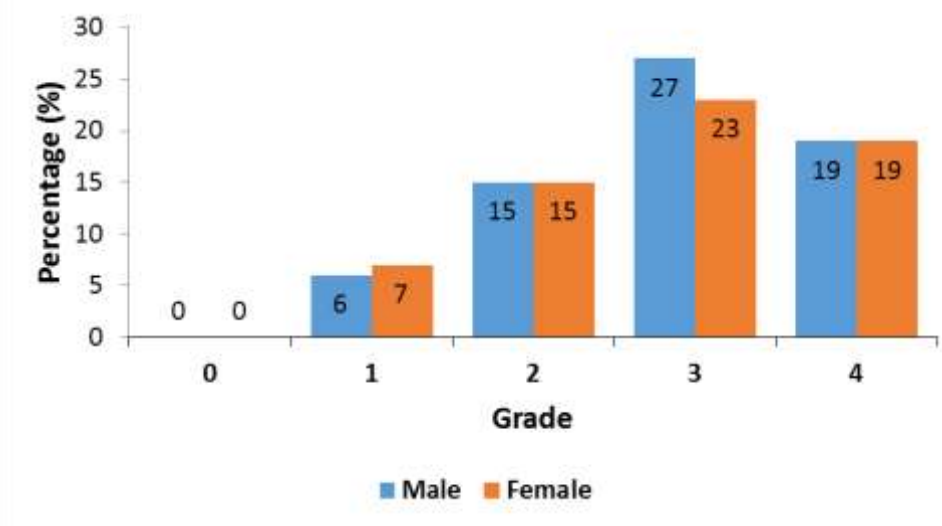


Figure 4.12 Variability in quality for IND/$ZnCl_2$ developed fingermarks on paper as a function of sex.

The grades given to fresh fingermark deposits indicate that there was a significant difference ($p = 2.7 \times 10^{-4}$, $Z = -3.638$) between donors over ($\mu_{1/2} = 2$, SD = 0.95) and under the age of 25 ($\mu_{1/2} = 3$, SD = 0.81), see Table 4.11. The Z score is much larger than the critical value ($Z = -1.96$), meaning that the null hypothesis can be rejected with greater than 99.99 % confidence. These findings are in agreement with the results of the pilot study, further reinforcing that differences in the amino acid content may exist as a function of donor age.

A significant difference was found due to the washing of hands within the hour prior to fingermark deposition ($p = 0.041$, $Z = -2.04$), and again reinforces the pilot results. The median values of donors who had washed their hands ($\mu_{1/2} = 3$, SD = 0.93) or not ($\mu_{1/2} = 3$, SD = 0.95) was significantly different and the null hypothesis can be rejected with 97.93 % confidence (Table 4.11). As amino acids are water soluble, the conclusion that lower grades (i.e. less reaction with IND/$ZnCl_2$) were given to donors who had washed their hands is very feasible.

No statistically significant dissimilarity was found between the grades given to treated fresh fingermarks from donors who had or had not recently consumed food (<1 hour, $p = 0.44$), applied cosmetics (<12 hours, $p = 0.65$) or due to the biological sex of the donor ($p = 0.87$), see Table 4.11. The food consumption and biological sex results once again mirror the pilot study findings.

Table 4.11 Statistical values gained from Mann-Whitney U tests, with the fresh luminescent fingermark grade given as a function of the independent variables.

Variable	Donor age		Washing of hands		Biological sex		Food consumption		Recent cosmetics (<12 hrs)	
	25 and over	Under 25	Yes	No	Male	Female	Yes	No	Yes	No
# of Donors	70	61	57	74	67	64	69	62	52	79
Median	3	3	3	3	3	3	3	3	3	3
Mean	2.57	3.20	2.68	3.00	2.85	2.87	2.81	2.92	2.81	2.90
Standard Deviation	0.99	0.79	0.93	0.95	0.95	0.96	0.94	0.96	1.03	0.90
U score	1384.0		1690.5		2109.5		1978.5		1963.0	
Z score	-3.638		-2.040		-0.167		-0.777		-0.449	
p-value	2.7×10^{-4}		0.041		0.868		0.437		0.653	

It is interesting to note that the trends found for the age ($p = 0.04$) and biological sex ($p = 0.46$) of the donor, as well as the recent use of cosmetics ($p = 0.35$), for 1 month old fingermarks agree with those of fresh fingermarks. The washing of hands ($\mu_{1/2} = 3$, $SD = 0.89$); however, did not have a significant impact on the grade given ($p = 0.085$, $Z = -1.72$) compared to donors who had not washed their hands ($\mu_{1/2} = 3$, $SD = 0.83$). This is in contrast to the findings with fresh fingermarks, yet the similarity of the median and mean scores (Table 4.12) indicates that both results were quite similar, where a 10 % higher average grade was given in both cases to samples deposited by donors who had not washed their hands.

While the effect of food consumption was found to be insignificant for grades given to both fresh and 1 month old fingermarks, the p value had decreased from 0.44 (fresh fingermarks) to 0.09 (older fingermarks). A longer timeframe for fingermarks to age could potentially result in a significant difference being found between donors who had and had not consumed food. As this trend was also observed in the pilot study, where it was proposed that chemical components present in food may increase the rate of Joullié's Pink degradation, food consumption may indeed cause faster decomposition of both reacted and unreacted amino acids.

Table 4.12 Statistical values gained from Mann-Whitney U tests, with the 1 month old luminescent fingermark grade given as a function of the independent variables.

Variable	Donor age		Washing of hands		Biological sex		Food consumption		Recent cosmetics (<12 hrs)	
	25 and over	Under 25	Yes	No	Male	Female	Yes	No	Yes	No
# of Donors	70	61	57	74	67	64	69	62	52	79
Median	3	3	3	3	3	3	3	3	3	3
Mean	2.69	3.06	2.82	3.10	2.99	2.97	2.84	3.13	2.87	3.05
Standard Deviation	0.92	0.77	0.89	0.83	0.97	0.74	0.92	0.78	0.95	0.80
U score	1708.0		1758.5		1993.5		1792.5		1864.5	
Z score	-2.086		-1.723		-0.734		-1.691		-0.944	
p-value	0.037		0.085		0.463		0.091		0.345	

4.4 Conclusions

The initial pilot study reinforced that variation of amino acids in sweat exists within a population and is detectable through examination of 1,2-indanedione treated latent fingermarks. Treatment with IND/$ZnCl_2$ allowed initial grading for fingermarks observed in both absorbance and luminescence mode. While 81 % of the samples achieved grades of 2 or above in luminescence mode initially, this decreased to 56 % after the treated samples aged 3 years. The Mann-Whitney U test and Wilcoxon signed rank test in conjunction with the Z score were utilised as statistical tools to assess fingermark grade variation. These tests indicated that grades of developed fingermarks vary significantly between fresh and aged fingermarks, the age of the donor and the washing of hands prior to deposition. Donors below the age of 25 offered superior grades, as did donors that did not wash the hands. However, the washing of hands prior to fingermark deposition was found to override the observed age of donor variation. No significant difference between the fingermark grade and food consumption or the sex of the donor was observed.

Following the initial pilot work, an investigation into the robustness and consistency of a fingermark grading method was conducted. The purpose was to evaluate its suitability in the large-scale donor project and to assess whether this grading scale is acceptable for fingermark development comparisons in general.

It was found that 67 % of the assessed fingermark images were graded consistent with the median score, and 99 % within one grade. The intraclass correlation coefficient was given as 0.973, indicating very strong agreement between all 11 graders for all scores given. Additionally, all fingermark graders were demonstrated to assign grades consistently for 78 % of duplicated images. The margin of error for the remaining duplicates was one grade. Wilcoxon signed rank tests indicated that there was no significant difference between the grades of the original and duplicated samples for any grader (mean $Z = -0.702$, mean $p = 0.53$). Overall, the grading scale was deemed an appropriate and consistent technique to acquire absolute values for developed latent fingermark samples, which can be used on its own or in combination with statistical methods to procure further knowledge. Furthermore, it was found that a small subgroup of graders did not differ significantly in their assessment from the larger group, indicating that this approach may be used in future work to avoid practical constraints in international collaborations.

Expanding the number of donors and the testing variables, complimentary data to the pilot study was found for the donor study. Wilcoxon signed rank tests again showed no significant difference between the original and duplicated grades for any of the 5 graders used in this study ($Z = -0.927$, $p = 0.38$). The intraclass correlation coefficient value of 0.847 also demonstrated that there was strong overall agreement between all 5 graders. In the donor study, a statistically significant difference was found between fingermark samples treated and graded within 3 days of deposition to those treated after one month; however, the actual distribution appeared to be very similar. Out of a total 1310 grades given, only 6 (0.5 %) returned a score of 0 and 64.6 % of all grades given were a 3 or 4. These tests indicated that grades of fingermarks developed within 3 days vary significantly between the age of the donor and the washing of hands prior to deposition. Donors who did not wash their hands the hour prior to deposition, or were below the age of 25, were more likely to offer higher grades. No significant variation between the fingermark grade and food consumption, sex of the donor or recent use of cosmetics was observed with fresh fingermarks.

Statistical analyses of samples that were treated after one month agreed with the results of the fresh fingermarks, except in the case of the washing of hands. Here, the Z score (-1.723) and p value (0.085) indicated that there was no dissimilarity between donors who had and had not washed their hands. It should also be mentioned that as with the pilot study, the food consumption had a greater effect for older fingermark deposits. While recent food consumption was not found to be statistically dissimilar in these studies, longer timeframes may potentially reveal that treated and untreated amino acids degrade faster after the consumption of food.

Chapter 5

Analysis of the amino acid content of latent fingermarks using High Performance Liquid Chromatography

5.1 Introduction

Variation in the response of amino acid sensitive fingermark treatment methods as an effect of the donor has become evident from Chapters 2, 3 and 4, as well as being published elsewhere [7, 202]. The work in Chapters 2 and 3 indicated that from a purely subjective point of view, there were strong and weak fingermark donors which influenced the development of ridge detail greatly. These findings encouraged the studies of Chapter 4, where treated and subsequently graded fingermark samples from large donor pools were statistically evaluated. Those results suggested that some variation exists between donors of different ages and previous activity. However, the non-specific nature of the fingermark reagents does not allow any further and more detailed chemical assessment of the findings relating to the donor effect. The need to more closely investigate the profiles of free amino acids in fingermark deposits, in order to allow for more meaningful statistics to be used, was recognised. The impetus was therefore to use a method that can be applied to a large sample population, is sensitive, selective, robust, and allows for absolute or relative quantification.

High performance liquid chromatography (HPLC) using UV detection has been well-established as a means for the analysis of amino acids [55, 75-77, 80, 203-205]. As the review by Deyl *et al.* outlines, free amino acids have been determined in a large variety of biological sources, including sweat, breast milk, saliva, hair, teeth, and liver cells [76]. As such, this approach has been shown to be sensitive, selective and reproducible for amino acid separation and detection.

Although various techniques exist for the separation of underivatised amino acids, these require an expensive specialised column and usually suffer from poor sensitivity compared to derivatised samples [76, 78, 203]. In essence, derivatisation agents react with the target analytes to offer reaction products which are more amenable to giving a detector response. The reaction of the derivatisation agent can be performed prior to the chromatographic separation (pre-column derivatisation), or afterwards (post-column derivatisation).

Post-column derivatisation, where the analytes are derivatised after separation to enhance their detectability, was first used in 1958 by Spackman *et al.* for the detection of amino acids using ninhydrin [206]. Some of the advantages of post-column derivatisation include reduced sample preparation, minimal interference from reaction artefacts and that the reaction does not have to go to completion [207]. However, this technique is not feasible for use with all analytes, especially with complex mixtures as the rate of reaction has to be fast, and long run times are required [207, 208]. This method also leads to band broadening as the addition of the reagent, the mixing of the solutions and the reaction rate cause dispersion. The instrument also becomes more complex and therefore more expensive to obtain and maintain [208, 209].

Aside from post column derivatisation, pre-column derivatisation can also be used to enhance analyte separation and sensitivity. Pre-column derivatisation using reversed-phase chromatography has emerged as the preferred choice for the separation of amino acids [76, 203]. Disadvantages of some pre-column derivatisation methods include the possible formation of more than one product, especially in complex samples, derivative instability, lack of reaction with secondary amino acids, as well as time consuming and extensive sample preparation (increasing the risk of human error and contamination) [207]. However, the reaction rate is not as important as with post-column derivatisation, the reaction conditions need not be compatible with the analysis conditions and it is a relatively inexpensive technique [80]. The analysis time is also reduced, and, depending on the derivatisation agent, the sensitivity is also much higher [80, 208, 209]. Some of the more commonly encountered derivatisation agents include *o*-phthaldialdehyde (OPA), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, butylisothiocyanate, dansyl chloride, 4-nitrophenylisothiocyanate, and phenylisothiocyanate (PITC) [77, 78, 207]. OPA derivatisation is very simple, sensitive and fast, but PITC is preferred when cysteine and secondary amino acid analysis is also required [79, 80]. The PITC derivatisation also yields more stable reaction products than OPA [210]. The reaction of PITC with amino acids is essentially the first step of the Edman degradation of peptides, and results in the formation of phenylthiocarbamyl amino acid deriviatives (Figure 5.1) [205, 211, 212].

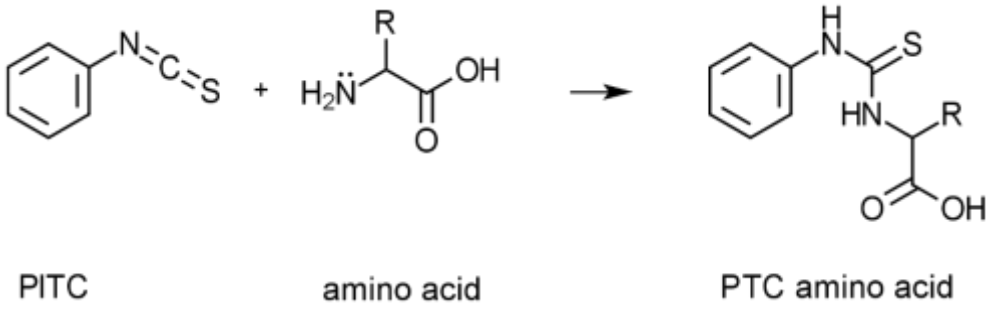


Figure 5.1 PITC reaction scheme with an alpha amino acid to form a phenylthiocarbamyl amino acid [212].

5.1.1 HPLC analysis of amino acids in latent fingermarks

The concentrations of dansyl chloride derivatised amino acids in fingermarks have been determined using HPLC coupled to a fluorescence detector, where the authors focussed on the comparison of fingermark deposits of healthy and beta-thalassemic donors [55]. Reliable differentiation between the two donor groups was found both qualitatively and quantitatively, especially with ornithine, lysine, proline and tyrosine (Figure 5.2). Serine, glycine, ornithine and alanine were again found to be the most abundant amino acids in the samples. Two columns were used in sequence for the complete separation of dansyl chloride derivatised samples. The method was fully validated and shown to be reliable, reproducible, and accurate. Degradation of derivatised samples was noted after 16 hours; however, all amino acids were found to be stable for three freeze and thaw cycles for one month, except for histidine and cysteine which were only stable for 10 days [55]. The major drawback of this method is the requirement for two columns to achieve satisfactory separation, as well as an overall elution time of 110 minutes, where tyrosine was the last to elute at 106 minutes. At a flow rate of 1 mL/min, this approach also uses an excessive amount of solvent.

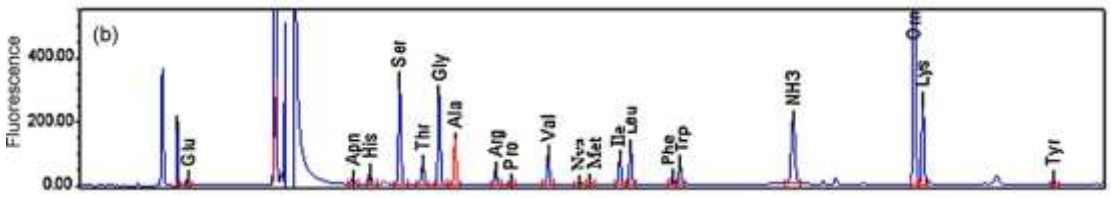


Figure 5.2 Fingermark sample chromatogram from Aala *et al.* [55].

5.1.2 Aims

This Chapter outlines the use of a HPLC with ultraviolet - diode array detector (HPLC-UV-DAD) for the analysis of 21 amino acids extracted from latent fingermark deposits. As described above, the analysis of amino acids using HPLC-UV has been well established. However, due to the large population size required for meaningful statistics, the analytical procedure needed to be simpler, quicker and suitable for amino acid analysis of fingermark residue extracted from a porous substrate [100]. A quick, efficient method for the collection and extraction of fingermark samples on a porous substrate was therefore required. A simpler method for the analysis of amino acids was vital to reduce the preparation and analysis time for the processing of large sample populations.

5.2 Materials and method

5.2.1 Chemicals

Acetonitrile (VWR International, USA), phenyl isothiocyanate (PITC, Sigma-Aldrich, USA), and triethylamine (TEA, Sigma-Aldrich, USA) were used as received and were of analytical reagent grade. MilliQ water (H_2O) was purified using an ion exchange system (IBIS Technology, Perth, Australia), followed by an Elga Purelab Ultra system with a 0.2 μm filter (Elga, High Wycombe, UK).

dl-2-Aminobutyric acid (Alfa Aesar, USA), dl-2,4-diaminobutyric acid (Alfa Aesar, USA), glycine (BDH, Australia), l-alanine (BDH, Australia), l-arginine (Sigma-Aldrich, USA), l-asparagine (Fluka Biochemika, Australia), l-aspartic acid (Sigma-Aldrich, USA), l-cysteine (Sigma-Aldrich, USA), l-glutamic acid (Sigma-Aldrich, USA), l-glutamine (Sigma-Aldrich, USA), l-histidine monochloride (BDH, Australia), l-isoleucine (Sigma-Aldrich, USA), l-leucine (Sigma-Aldrich, USA), l-lysine (Sigma-Aldrich, USA), l-methionine (Sigma-Aldrich, USA), l-norleucine (Sigma-Aldrich, USA), l-ornithine monohydrochloride (Sigma-Aldrich, USA), l-phenylalanine (Sigma-Aldrich, USA), l-proline (Sigma-Aldrich, USA), l-serine (Sigma-Aldrich, USA), l-threonine (Sigma-Aldrich, USA), l-tryptophan (Sigma-Aldrich, USA), l-tyrosine (Fluka Biochemika, Australia) and l-valine (Sigma-Aldrich, USA) were of analytical reagent grade or above. The simulant pads were amino acid based Latent Print Reference Pads (Forensic Source, USA).

5.2.2 Sample collection

50 donors were asked to rub their hands together and then to firmly and evenly press one finger on an individual filter paper (Whatman® No.1, Ø 25 mm) for 10 seconds, where impressions of the index, middle and ring finger were attained. The fingermarks were collected as uncharged deposits, and donors were not asked to wash their hands prior to analysis. The samples were wrapped in aluminium foil, and if the immediate analysis was not possible they were stored at -20 °C. For each donor, a survey questionnaire was also collected in the event that any of the listed factors may have had a significant impact on any statistical modelling to be applied (Table 5.1). As for Chapter 4, this form requested information such as the donor's age, biological sex, activity and personal hygiene (Appendix A). Amino acid simulant samples were deposited onto filter paper using a rubber fingerprint stamp.

Table 5.1 Donor information with regards to the number of donors for each variable (n=50).

Variable	Grouping	Number of Donors
Biological sex	Male	*29*
	Female	*21*
Age	Over 25	*23 (M:16, F:7)*
	Under 25	*27 (M:13, F:14)*
Food Consumption	Yes	*16*
	No	*34*
Washing of hands	Yes	*10*
	No	*40*
Cosmetics	Yes	*16*
	No	*34*

5.2.3 Sample preparation

Amino acid standards were prepared in water, except for l-aspartic acid which was dissolved in 50 % (v:v) methanol:water; and l-tyrosine which was dissolved in 0.1 M hydrochloric acid in 30 % (v:v) methanol:water. All amino acids were made up at a concentration of 1 g/L individually. These were then used to prepare a stock solution mixture containing 21 amino acids, each at a concentration of 40 ng/µL. An internal standard (IS) solution was made up of dl-2-aminobutyric acid and l-norleucine at a concentration of 40 ng/µL in water. These standards were used for all method

development and validation purposes and kept refrigerated at 4 °C to reduce degradation, unless otherwise stated.

Samples were extracted from the filter paper by soaking in 1 mL of 50 % (v:v) methanol:water for one hour. Extracts (300 µL) were syringed into 2 mL screw top chromatography vials and 10 µL of the IS mixture was added and subsequently evaporated to dryness under nitrogen gas. Due to equipment availability, the samples were dried using a Dry Block Heater (Model DBH30DP, Ratek, Australia) under nitrogen gas (without temperature control), instead of being dried under high vacuum as used by previous authors [203, 204]. In the case of standard solutions, the required concentration was syringed into vials together with 10 µL of the IS mixture and subsequently evaporated to dryness under nitrogen gas.

Derivatisation agent consisting of ethanol:water:TEA:PITC (7:1:1:1) was prepared fresh each day, where PITC was added under nitrogen. 20 µL of the derivatisation agent was added to the dried samples under nitrogen and sealed for 30 minutes at room temperature. The reagents were then evaporated under nitrogen gas. The samples were reconstituted with 30 µL of mobile phase B and vortex mixed (see Table 5.3 for mobile phase composition). The samples were finally made up to 300 µL with mobile phase A (to reduce the fronting effect which can occur when the mobile phase equilibrium in the column in disturbed) and vortex mixed prior to injection [80].

5.2.3.1 Method development and simplification

For all method development, simplification and validation experiments, the concentration of each amino acid and IS was 0.8 ng/µL, unless otherwise specified. Extractions using 30 or 50 % (v:v) methanol:water compositions were trialled. For each solvent composition, samples were also either extracted without (30 or 60 minutes) or with agitation (5 or 10 minutes) using an ultrasonic bath (8891 Sonicator, Coler-Parmer, USA).

The TEA pre-derivatisation step was investigated as described in Table 5.2. The amount of derivatisation agent required for maximum sensitivity was tested by reacting the amino acid mixture (4 ng/µL) with 20, 40, 60, 100, 150 and 200 µL amounts of the derivatisation mixture. Injection volumes of 10, 20, 50 and 100 µL, and 2 mL and 250 µL vial inserts, were trialled to increase the sensitivity of the method. The drying step after derivatisation was considered, where samples were analysed with and without the nitrogen gas drying step. Where the derivatisation agent was not dried, the amount of mobile phase A was reduced by 20 µL to maintain the same sample volume.

Table 5.2 TEA pre-derivatisation experiments for sample preparation.

Method	Conditions
Method 1	Analyte dried under nitrogen gas, 20 µL ethanol:water:TEA (2:2:1) was used to reconstitute, sample dried again prior to derivatisation [80]
Method 2	10 µL ethanol and 5 µL TEA were added directly to the wet sample, then dried prior to derivatisation
Method 3	Omission of the TEA pre-derivatisation step

Mixed calibration standards of the 21 amino acids were prepared by the serial dilution of the stock mixture with 50 % (v:v) methanol:water. All calibration standards were run in triplicate covering a concentration range of 0.002 – 2 ng/µL. 10 standard solutions and 10 extracted standards were used for instrument precision and extraction efficiency determinations.

5.2.4 HPLC instrumentation

The LC used was an Agilent 1200 HPLC system (Palo Alto, USA), and solvent was degassed by manually passing it through a 0.45 µm pore, 47 mm filter (Phenex™ Teflon® Filter Membranes, Phenomenex, USA). The system was additionally fitted with a degasser unit and flow was achieved using a 1260 series Infinity binary pump. Samples were placed into a 100 well-plate autosampler which was kept refrigerated at 4 °C to reduce sample degradation. An Agilent (Palo Alto, USA) XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size) was used at a flow rate of 0.8 mL/min in the elution program, unless stated otherwise. The diode array detector was set to 254 nm and the column temperature remained at 41 °C unless stated otherwise. Further LC conditions and parameters are detailed in Table 5.3.

Table 5.3 LC parameters for the separation of amino acids.

Time (mins)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)	LC conditions
0.00	90	10	0.8	Eluent A: 50 mM sodium acetate in MilliQ water, adjusted with 0.4 mL/L triethylamine and 0.1 % acetic acid Eluent B: 60 % acetonitrile in MilliQ water Column: Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size) Injection volume: 100 µL Column temperature: 41 °C
3.00	90	10	0.7	
7.00	87	13	0.7	
9.00	80	20	0.7	
12.00	78	22	0.7	
12.50	70	30	0.9	
14.00	60	40	0.9	
16.50	62	38	0.7	
18.00	53	47	1.0	
20.00	58	42	0.8	
22.00	35	65	0.9	
23.00	0	100	0.9	
30.00	0	100	0.9	
31.00	90	10	0.8	
45.00	90	10	0.8	

The peak areas and heights of the analytes were calculated using Agilent ChemStation (version B.03.02). Integration was performed as per the standard software parameters (baseline correction changed to advanced mode) and manual baseline correction was carried out where needed.

5.2.5 Statistical analyses

The Mann-Whitney U test was performed with IBM's SPSS Version 2.0. Principal component analysis (PCA) was performed with the Unscrambler® X 10.3 Software (CAMO Software AS, Oslo, Norway).

5.3 Results and discussion

5.3.1 Method development

A recent procedure outlined by Gheshlaghi *et al.* used a statistical approach to optimise the analysis of free amino acids in an aqueous matrix using HPLC-UV [80]. Their method was selected as a starting point due to good separation of the 20 standard amino acids, reasonable elution times and relatively straight-forward sample preparation [80]. Gheshlaghi's method was used to obtain the retention times of the 20 standard amino acids and l-ornithine. Two signals were observed for cysteine, where the minor peak (quicker elution) is thought to be the single cysteine

phenylthiocarbamyl derivative, and the major peak the cystine dimer (two cysteine molecules joined by a sulfur bridge). 3 internal standards (l-norleucine, dl-2,4-diaminobutyric acid and dl-2-aminobutyric acid) were also added to the method.

The analysis of the combined amino acid and IS mixture resulted in the co-elution and incomplete separation of dl-2,4-diaminobutyric acid, ornithine and tryptophan [80]. The IS dl-2,4-diaminobutyric acid was subsequently omitted from the method, yet ornithine and tryptophan were still not baseline resolved.

A less recent publication by Hariharan *et al.* presented chromatographic conditions resulting in the separation of 26 phenylthiocarbamyl derivatives of amino acids [203]. However, the first eleven amino acids were not as well resolved as with the method by Gheshlaghi *et al.* [80]. The main differences between the two methods were a more acidic pH (5.10 vs. 6.10) of mobile phase A (50 mM sodium acetate in MilliQ water, adjusted with 0.4 mL/L triethylamine and ~0.05-0.1 % acetic acid), different elution program and a lower column temperature (41 °C vs. 44 °C) of Hariharan's method [203]. Based on these parameters, a combination of the two methods was further investigated to obtain improved analyte separation.

Only slight improvements to the ornithine and tryptophan separation were observed by adapting the elution program to enhance the separation of the more strongly retained analytes using a more polar gradient program after 14 minutes [203]. Lowering the column temperature had a more positive effect, where the overall separation was increased. However, ornithine and tryptophan still co-eluted to some extent.

The pH of mobile phase A was reduced stepwise, where ornithine and tryptophan could be fully resolved using a pH of 5.10 (using 0.1 % (v/v) acetic acid), as per Hariharan *et al.* [203]. Due to the lower pH, the separation of some of the more polar amino acids had suffered, and the elution program was changed in tandem with the flow rate. A range of flow rates, from 0.6 to 1.0 mL/min, at different times in the elution program were adopted in favour of the isocratic 0.9 mL/min (Table 5.3). By changing the parameters discussed above, good separation of all 21 amino acids and the two IS was achieved (Figure 5.3). Please note that the first five minutes of the

chromatograms in this study only showed the solvent front, to improve the visibility of the relevant signals the chromatograms were therefore truncated. A summary of the retention times can be found in Table 5.4. A typical fingermark sample chromatogram obtained by using these conditions is presented in Figure 5.4. Despite the good chromatographic separation, the overall procedure still needed to be more sensitive and required simplification for the analysis of large numbers of fingermark samples.

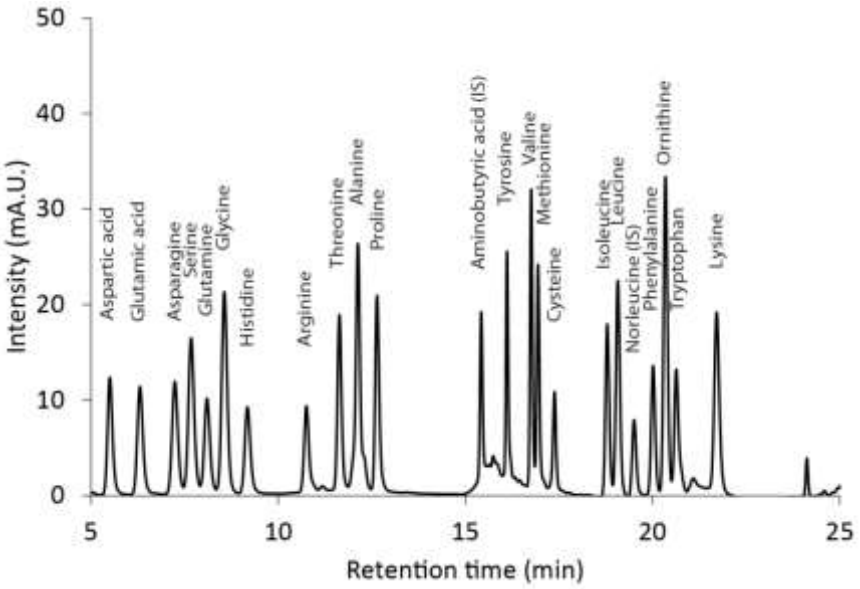


Figure 5.3 Typical chromatogram of a PITC derivatised stock solution containing 21 amino acids and 2 IS. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

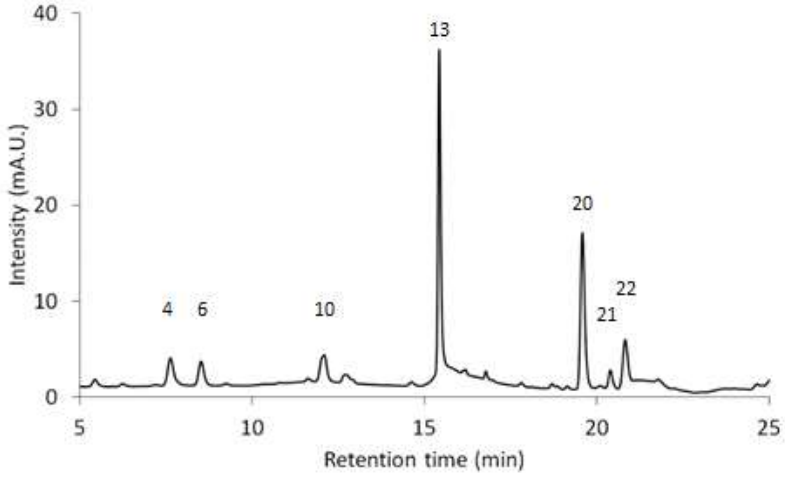


Figure 5.4 Sample chromatogram of a PITC derivatised fingermark extract prior to method improvements. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm. For the amino acids corresponding to the numbers, refer to Table 5.4.

Table 5.4 Retention times of the 21 amino acids and 2 internal standards using new chromatographic conditions.

Amino acid number	Amino acid	Retention time (min)
1	Aspartic acid	6.44
2	Glutamate	7.38
3	Asparagine	8.30
4	Serine	8.80
5	Glutamine	9.28
6	Glycine	9.84
7	Histidine	10.67
8	Arginine	12.29
9	Threonine	12.97
10	Alanine	13.44
11	Proline	13.88
12	Cysteine (minor)	15.91
13	Aminbutyric acid (IS)	16.17
14	Tyrosine	16.97
15	Valine	17.65
16	Methionine	17.81
17	Cysteine (major)	18.21
18	Isoleucine	20.04
19	Leucine	20.34
20	Norleucine (IS)	20.69
21	Phenylalanine	21.04
22	Ornithine	21.25
23	Tryptophan	21.54
24	Lysine	22.57

5.3.2 Method improvement

In order to simplify the sample preparation procedure, and make the analysis more sensitive, the procedure outlined in Table 5.5 was investigated. Due to equipment availability, the drying steps were performed using nitrogen gas flow rather than a vacuum chamber from the outset of this study.

Table 5.5 Comparison of the standard sample preparation procedure by Gheshlaghi *et al.*, and the simplified procedure established in this study [80].

Standard method [80]	Steps	New method
N/A	***Sample deposition***	Filter paper circles
N/A	***Extraction***	1 hour soak with 50 % (v:v) methanol:water
Under vacuum	***Sample drying***	With nitrogen gas
20 µL of ethanol:water:TEA (2:2:1 v:v)	***Pre-derivatisation***	-
Under vacuum	***Sample drying***	-
20 µL of ethanol:water:TEA:PITC (7:1:1:1 v:v)	***Derivatisation***	20 µL of ethanol:water:TEA:PITC (7:1:1:1 v:v)
Under vacuum	***Sample drying***	With nitrogen gas
500 µL of mobile phase	***Reconstitution***	300 µL of mobile phase
10 µL injection volume	***Analysis***	100 µL injection volume

5.3.2.1 Sample deposition

Previous studies into the chemistry of fingermark deposits used substrates such as glass vials and beads, polypropylene vials, Mylar® films and cotton gloves for fingermark collection [32, 35, 68, 69, 72]. These approaches are not ideal for the analysis of large numbers of latent fingermarks, as their extraction methods and/or media pre-treatment makes them either time consuming and/or expensive. A colleague's recent use of filter paper for the collection and subsequent extraction of fingermark lipids indicated this to be a cheap and convenient collection medium, while also mimicking the porous nature of exhibits treated with amino acid sensitive reagents [213].

5.3.2.2 Extraction

Various agitation and methanol compositions were tested to find a quick, efficient and reproducible amino acid extraction procedure for fingermarks deposited on filter paper. Amino acid stock solutions were spotted onto filter paper and extracted using 1 mL of 30 and 50 % (v:v) methanol:water solutions. Samples were sonicated for either 5 or 10 minutes and compared to those that soaked for either 30 or 60 minutes without sonication.

As expected, the particular polarity of each amino acid dictated the preferred extraction method (see Table 5.6) [214]. On average, the extraction methods using sonication resulted in a much larger variation than those without sonication (see Appendix B). Samples extracted for 1 hour in 50 % (v:v) methanol:water without sonication provided consistent results with the highest average efficiency, especially for more polar analytes.

Table 5.6 Relative extraction efficiencies, normalised to the highest extraction value of each amino acid, of the various extraction methods (%).

	Extraction efficiency (%)							
	30 % (v:v) methanol:water				**50 % (v:v) methanol:water**			
Amino acid	**Agitation 5 mins**	**Agitation 10 mins**	**No agitation 30 mins**	**No agitation 60 mins**	**Agitation 5 mins**	**Agitation 10 mins**	**No agitation 30 mins**	**No agitation 60 mins**
Aspartic acid	88.3	79.4	100.0	99.2	77.3	86.4	88.8	90.0
Glutamate	87.0	82.5	100.0	99.2	83.9	96.4	95.2	99.7
Asparagine	85.0	81.4	98.6	98.3	80.2	97.2	95.4	100.0
Serine	88.3	80.5	97.5	97.0	83.4	97.9	95.2	100.0
Glutamine	85.9	80.3	97.5	96.4	83.0	97.8	94.9	100.0
Glycine	95.3	79.9	95.3	95.6	94.2	98.0	94.3	100.0
Histidine	86.1	73.2	89.4	82.1	91.0	89.1	87.1	100.0
Arginine	84.5	78.7	89.9	90.5	88.6	100.0	92.9	98.2
Threonine	88.8	80.3	96.0	93.8	84.8	96.9	93.5	100.0
Alanine	88.9	94.3	96.7	96.4	87.1	96.3	93.7	100.0
Proline	100.0	76.2	92.5	91.0	96.3	93.9	89.8	94.7
Cysteine (minor)	95.5	83.7	99.4	97.2	90.1	97.6	96.8	100.0
Aminbutyric acid (IS)	97.4	83.4	97.3	95.5	96.8	98.0	95.3	100.0
Tyrosine	96.3	82.6	97.5	96.2	90.3	96.2	93.8	100.0
Valine	95.4	72.9	93.6	89.8	91.8	94.2	89.5	100.0
Methionine	61.5	85.6	80.9	91.6	91.5	98.3	100.0	93.5
Cysteine (major)	100.0	79.6	97.4	92.7	93.3	93.6	91.1	97.1
Isoleucine	100.0	80.8	96.7	94.3	91.9	94.4	91.5	98.5
Leucine	100.0	77.3	97.7	91.0	87.4	89.5	89.2	92.5
Norleucine (IS)	100.0	73.7	91.4	86.1	89.9	87.1	84.8	90.0
Phenylalanine	98.7	85.0	100.0	98.7	91.4	95.5	94.2	98.2
Ornithine	100.0	91.1	100.0	88.8	78.5	73.9	76.6	79.0
Tryptophan	100.0	82.1	96.1	92.3	90.3	91.8	90.2	93.5
Lysine	88.3	79.4	100.0	99.2	77.3	86.4	88.8	90.0
Average	92.1	81.0	95.9	93.9	87.9	93.6	91.8	96.5

5.3.2.3 Pre-derivatisation solution

A "redry" [215] or pre-derivatisation [216] solution consisting of ethanol:water:TEA (2:2:1 v/v), is commonly employed prior to derivatisation with PITC [75, 80, 203, 217]. This step serves to form deprotonated amines, increasing the rate of reaction of derivatisation [218]. As PITC is added to the analyte in a mixture already containing TEA, this may render the additional redry step unnecessary (Figure 5.1). Three different approaches to the pre-derivatisation step were compared to simplify the sample preparation procedure (Table 5.7). The analyte separation and intensity appeared to be largely unaffected by the three different methods and given the similarity in the results, Methods 1 and 3 were compared in more detail (Figure 5.5).

Table 5.7 Comparison of three different pre-derivatisation approaches.

Method	Pre-derivatisation solution	Pre-derivatisation drying step
1 (standard method) [80]	Yes	Yes
2	Yes	No
3	No	No

A slight shoulder on the alanine peak, lower intensity of tryptophan and improved baseline in chromatograms recorded using Method 3 appeared to be the main differences (Figure 5.5). Due to the similar performances, Method 3 was used exclusively for subsequent analyses as this significantly reduces sample preparation time and chances of contamination and human error.

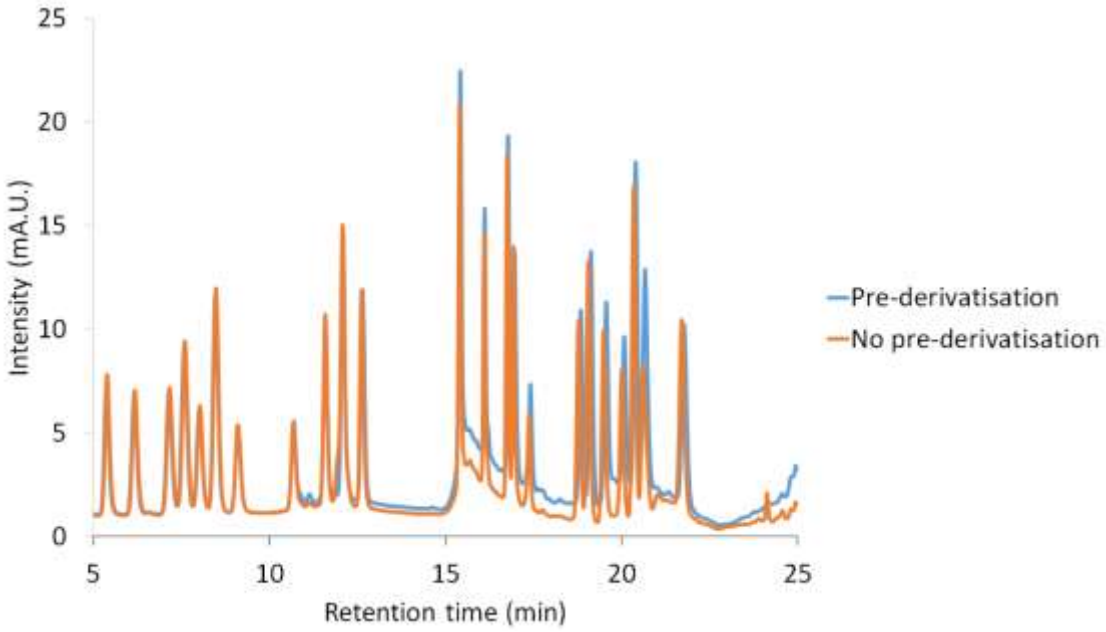


Figure 5.5 Analysis of PITC derivatised amino acid stock solution and IS mixture, comparing the pre-derivatisation approaches, where the standard procedure is represented by Method 1 (pre-derivatisation) [80] and Method 3 omits the pre-derivatisation steps. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

5.3.2.4 Sample drying steps

To further simplify the sample preparation process, removal of the drying step prior to derivatisation was investigated. However, the reagent was only partially soluble when added directly into the sample dissolved in 50 % (v:v) methanol:water. This resulted in reduced signal intensity and the appearance of unknown peaks, hence the step could not be omitted.

According to Heinrikson and Meredith, the post-derivatisation drying step removes a peak that is an artefact arising from analytes dissolved in hydrochloric acid [204]. Out of 23 analytes used in this study, only tyrosine was made up in 0.1 M hydrochloric acid, and this post-derivatisation drying step was omitted. Compared to the standard procedure, no significant difference was initially found in the chromatograms. As the sample preparation is much quicker (drying the derivatised analytes can take several hours for numerous samples), this simplified approach was intended to be adopted for all future work. After several weeks, however, it was noted that 2 unknown peaks had started occurring in the chromatograms (at 12.69 and 20.79 minutes, Figure 5.6). Initially, it was thought that contamination due to

either TEA or PITC degradation was responsible, yet fresh reagents did not alleviate the issue [75]. The gradient elution program and the flow rate were revised to minimise the co-elution with the desired analytes. The intensity of the first contaminant was time dependent, where an increase in intensity was noted following extended sample runs. The post-derivatisation drying step was revisited, where a reduction of the first contaminant in dried samples indicated that the it was probably due to the ethanol:water:TEA:PITC mixture. The post-derivatisation drying step was therefore re-introduced, which may also minimise the increased column deterioration noted by Fürst *et al.* with 'wet' samples, although this was not observed in this study [219]. The second contaminant could not be removed entirely, and was present throughout the remainder of the project, but did not appear to affect fingermark analysis unduly (Figure 5.6).

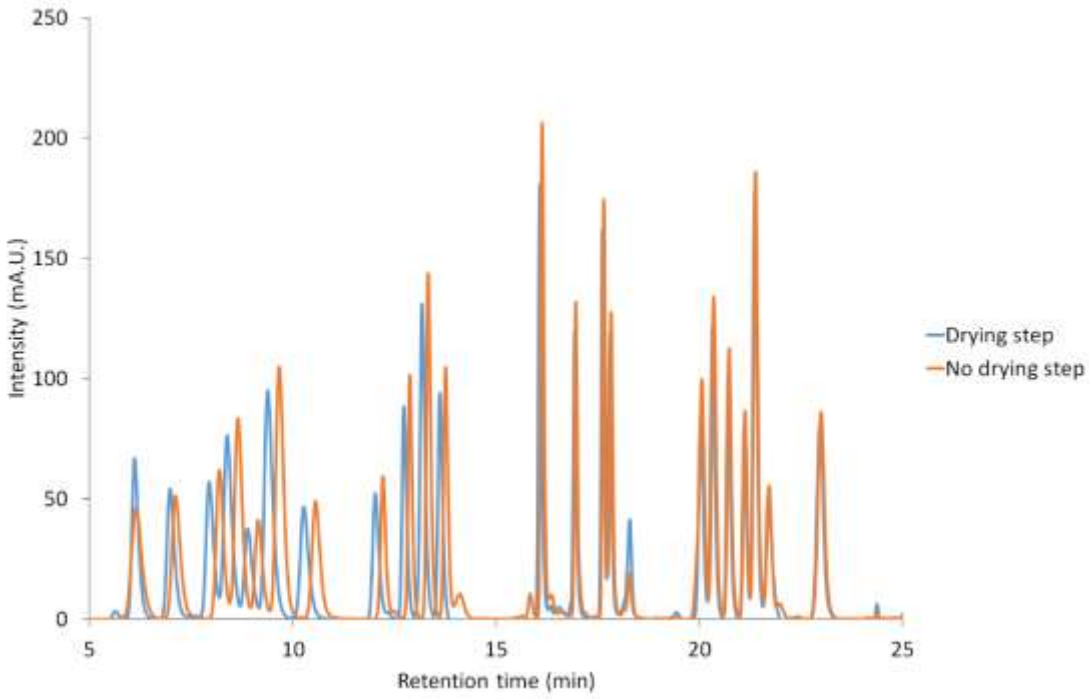


Figure 5.6 Comparison of the PITC derivatised amino acid stock solution and IS mixtures, where the drying step prior to derivatisation was investigated. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

5.3.2.5 Derivatisation reagent concentration

To determine whether sufficient PITC was present for complete derivatisation of the amino acids, a concentrated amino acid mixture (4 ng/µL) was reacted with increasing amounts of the derivatisation reagent. As per previous publications, the smallest volume (20 µL) of derivatisation reagent was used as it appeared to give the highest signal intensity in combination with a slightly faster elution time (Figure 5.7) [80, 203]. These results suggest that even with very concentrated samples, the amino acids are the limiting reactants and not the derivatisation agent.

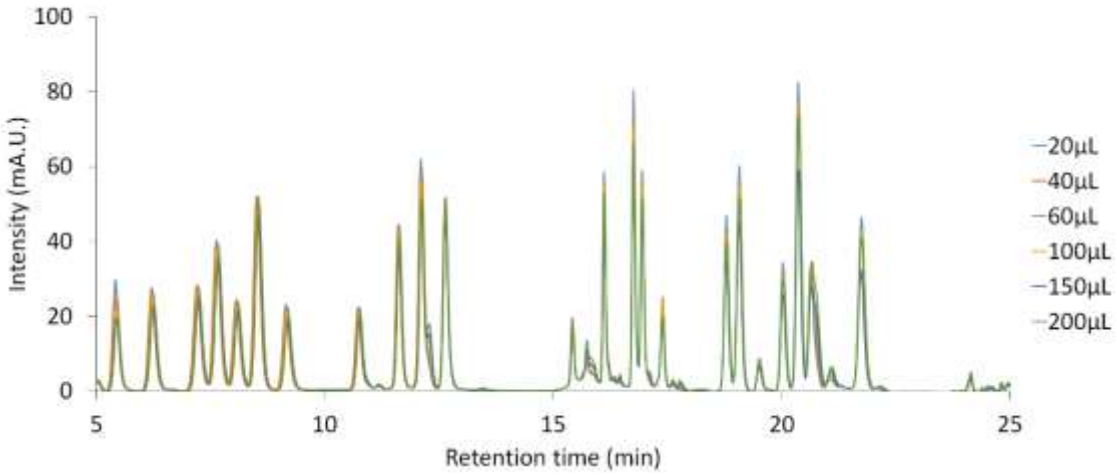


Figure 5.7 Comparison of chromatograms of concentrated amino acid mixtures (4 ng/µL) and IS' reacted with increasing volumes of the PITC derivatisation agent. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

5.3.2.5 Sensitivity

To increase the sensitivity of the analysis, changes to the 10 µL injection volume and reductions to the 500 µL final sample volume were investigated. Larger injection volumes (20, 50 and 100 µL) resulted in a linear increase in the detector response (Figure 5.8).

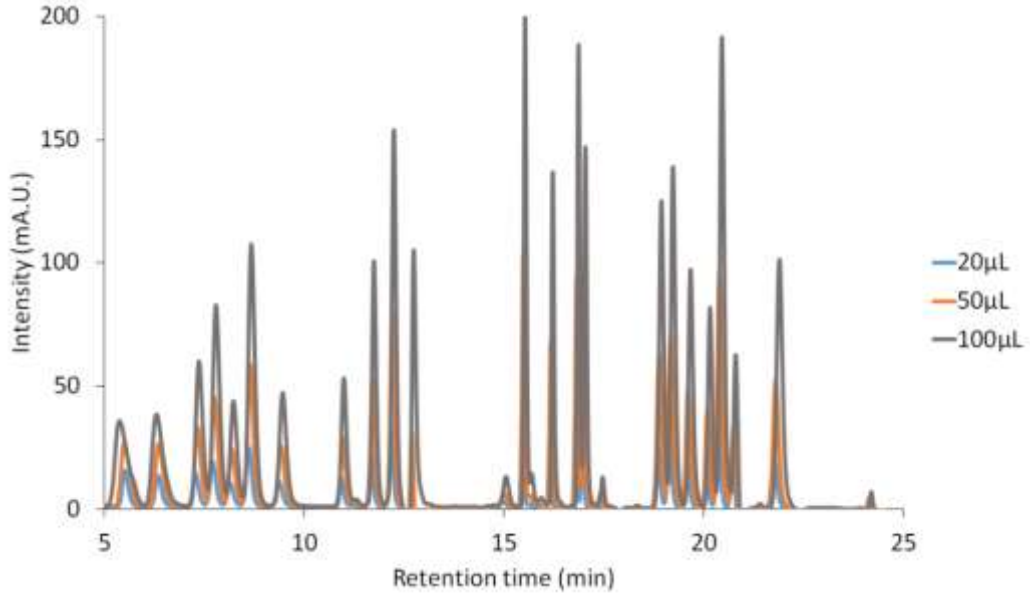


Figure 5.8 Chromatograms illustrating the increase in detector response due to larger injection volumes of the PITC derivatised amino acid mixture and IS solutions. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

Micro-inserts for chromatography vials allow for smaller sample volumes, and therefore more concentrated samples, to be analysed. Vials with micro-inserts (150 µL sample volume and 20 µL injection volume) were compared to regular 2 mL vials (300 µL sample volume and 100 µL injection volume), as seen in Figure 5.9. The vials with micro-inserts resulted in more intense signals, which could be further improved by using yet larger fill (200 µL) and injection volumes (100 µL).

However, practical issues were encountered with the micro-inserts, where less consistent results were recorded in addition to the higher material cost. It was postulated that the nitrogen gas used for the drying steps may have caused some of the sample to be expelled. Therefore, 2 mL vials were used with increased fill and injection volumes of 300 and 100 µL, respectively.

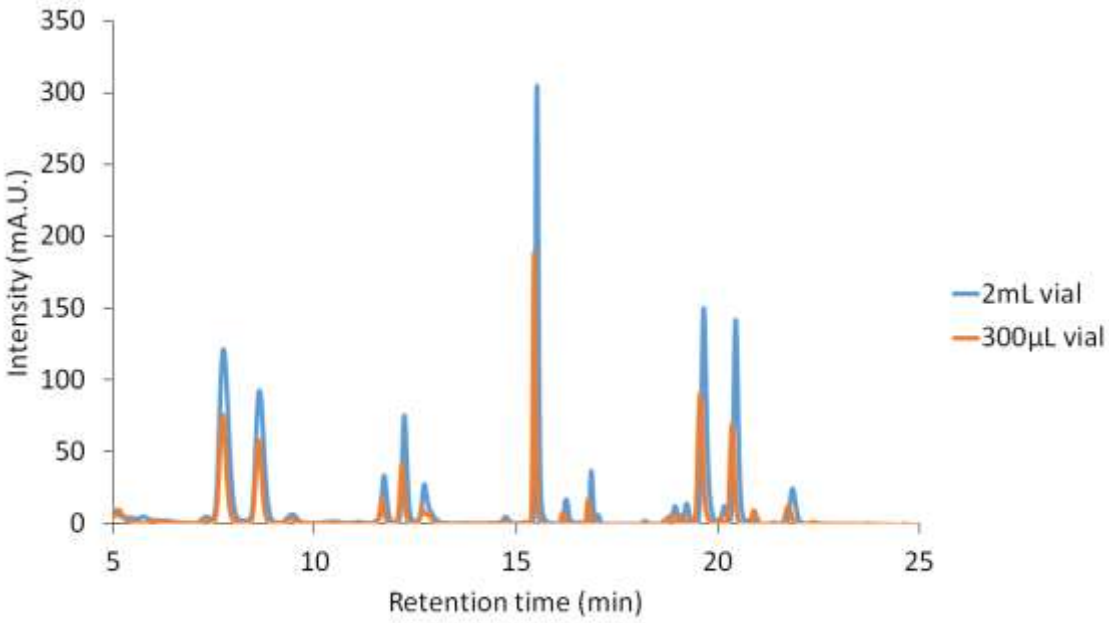


Figure 5.9 Chromatograms of PITC derivatised amino acid mixture and IS solution, highlighting the variation in signal intensity when comparing samples made up in 2 mL vials (fill and injection volumes of 300 and 100 uL, respectively) and vials with a micro-insert (fill and injection volumes of 200 and 100 uL, respectively). Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

The combination of all aspects of the method development yielded separation of 21 amino acids and 2 IS, with a level of sensitivity that was appropriate for the analysis of fingermark samples extracted from a porous substrate. Furthermore, the sample preparation step had been significantly simplified to make this approach more amenable to the analysis of a large population size, as suggested by the International Fingerprint Research Group [100]. To establish the sensitivity, reproducibility and limitations of the developed procedure, the method was validated as outlined below.

5.3.3 Method validation

Method validation was performed according to the guidelines provided by Peters *et al.* [220]. The signal to noise ratio was used to estimate the limit of detection, LOD, (S/N > 3) and lower limit of quantification, LLOQ, (SN > 10) (Table 5.8). The linear calibration range for the method was established to lie within 0.002 and 2 ng/µL for IS adjusted data, where the average correlation coefficient was found to be 0.993 (calculated using peak area) and 0.992 (calculated using peak height) (Table 5.8). Histidine, alanine and tryptophan were quantified at concentrations of 0.01 ng/µL or higher. Cysteine was only quantified at concentrations above 0.2 ng/µL. Prior to the

method improvements outlined in the previous section, the average correlation coefficient for the IS adjusted calibration was slightly better at 0.995, but the working range was only from 0.04 to 2 ng/µL.

Good precision, a measurement of the reproducibility and variation (scatter) of measurements due to random error, was displayed within 10 replicates. The precision of standard solutions spotted directly into the vial was found to be better than the extracted samples. Values better than the acceptable limit of the relative standard deviation (RSD) of 15 % was achieved for all amino acids extracted from filter paper (Table 5.8) [220]. Extraction efficiencies were calculated by spotting and extracting the amino acid stock solution from 10 filter papers, in comparison to 10 samples which were spotted directly into sample vials. After accounting for the sample volume, the average extraction efficiency was shown to be 82.4 %, where the type of amino acid determined its exact recovery (Table 5.8). Some amino acids were extracted with higher than 100 % efficiency. As analyses of blank filter paper showed negligible amino acid contamination, it was postulated that a decrease in the sample volume due to absorption by the filter paper may cause some amino acids to be more concentrated than expected. Alternatively, the difference could simply be due to the RSD (error) in the measurements.

It was found that derivatised samples showed signs of degradation within 4 weeks of preparation (data not shown). Underivatised stock solutions, on the other hand, could be stored for extended periods of time without appreciable degradation, as per previous publications [80, 203]. Fresh and one year old amino acid standard solutions (stored at 4 °C) were compared and found to be very similar, where only cysteine showed signs of degradation, possibly due to oxidation.

Table 5.8 Validation data for various aspects of the method validation procedure.

Amino acid	Correlation coefficient (0.002-2 ng/µL)	Limit of detection (pg/µL)	Lower limit of quantification (pg/µL)	Precision (% RSD)	Extraction efficiency (%)
Aspartic acid	1.000	0.83	2.78	4.84	85.73
Glutamate	1.000	0.91	3.02	5.16	85.18
Asparagine	0.996	0.57	1.9	4.03	88.88
Serine	0.989	0.46	1.54	4.40	89.69
Glutamine	0.977	0.78	2.61	4.42	90.79
Glycine	0.991	0.31	1.02	4.31	94.50
Histidine	1.000*	3.12	10.39	2.93	66.84
Arginine	1.000	0.76	2.55	3.37	74.20
Threonine	1.000	0.4	1.33	4.98	82.67
Alanine	0.999*	0.38	1.28	6.39	105.81
Proline	1.000	0.39	1.3	10.56	78.12
Aminbutyric acid (IS)	-	-	-	6.34	84.05
Tyrosine	0.991	0.24	0.81	3.86	60.50
Valine	1.000	0.24	0.8	4.64	90.96
Methionine	1.000	0.29	0.96	4.22	73.71
Cysteine (major)	0.910*	6.73	22.43	13.31	58.78
Isoleucine	1.000	0.13	0.42	7.88	71.48
Leucine	1.000	0.26	0.88	4.75	89.82
Norleucine (IS)	-	-	-	8.58	95.45
Phenylalanine	1.000	1.13	3.75	8.73	104.81
Ornithine	0.999	0.2	0.66	3.28	71.17
Tryptophan	1.000*	0.71	2.36	10.94	84.83
Lysine	0.999	1.29	4.29	5.37	68.21
Average	*0.993*	*0.96*	*3.19*	*5.97*	*82.44*

***NB:** Histidine, alanine and tryptophan were quantified at concentrations higher than 0.01 ng/µL. Cysteine was quantified at concentrations above 0.2 ng/µL.

5.3.4 Fingermark sample analysis

The method developed above was then applied to investigate the amino acid content of latent fingermarks deposited on filter paper by 50 donors. Prior to the application of any statistics, some general trends could be discerned by visual inspection of the chromatograms (Figure 5.10). As per the collated data presented in the review by Girod *et al.* [30], it was found that serine was the most abundant amino acid in all samples (Table 5.9), followed by glycine, ornithine, alanine, and aspartic acid (in that order). The average concentration of each amino acid per fingermark was found to be between 1.2 ng (glutamine) and 190.2 ng (serine), Table 5.9. The sum of the average amino acid concentrations was shown to be 675 ng per fingermark, but ranged from

40 to 8050 ng per fingermark depending on the donor. Croxton *et al.* found the average amino acid content in 18 donors to be between 20.7 and 345.1 ng per fingermark (with a more limited number of amino acids) [34], Hansen *et al.* gave an estimation of 250 ng per fingermark [103] and Bayford a range of 0.3 to 2.59 mg/L [221]. It is unclear as to why the values found in this study are so much higher than the concentrations found in the literature, but differences in the collection, extraction, instrumental procedure and the local donor population may account for some of the variation, and more amino acids were investigated in this work. This study also investigated a much larger number of donors (50) than previous work by Croxton *et al.* (18), Atherton *et al.* (2) and de Puit *et al.* (20), which should equate to a more representative donor population [34-36].

Table 5.9 Abundance of amino acids, both absolute values and relative to the sum of total amino acid concentration, of donor traits.

Amino acid	Absolute concentration (ng/fingermark)					Relative concentration (%)				
	All donors	Female donors	Male donors	Under 25	25 and over	All donors	Female donors	Male donors	Under 25	25 and over
Aspartic acid	36.9	25.8	45.0	52.8	18.0	5.5	5.3	5.5	5.5	5.3
Glutamate	15.3	10.5	18.6	22.2	7.2	2.3	2.2	2.3	2.3	2.1
Asparagine	6.6	4.8	7.8	9.0	3.6	1.0	1.0	1.0	1.0	1.0
Serine	190.2	135.3	230.1	281.1	83.7	28.2	28.0	28.3	29.3	24.7
Glutamine	1.2	0.9	1.2	1.8	0.6	0.2	0.2	0.2	0.2	0.1
Glycine	93.6	64.8	114.6	137.7	42.3	13.9	13.4	14.1	14.3	12.4
Histidine	34.8	24.0	42.9	56.4	9.6	5.2	4.9	5.3	5.9	2.9
Arginine	29.7	19.2	37.5	38.7	19.2	4.4	4.0	4.6	4.0	5.6
Threonine	26.4	18.9	31.8	35.4	15.9	3.9	3.9	3.9	3.7	4.7
Alanine	48.6	39.0	55.5	67.2	26.7	7.2	8.1	6.8	7.0	7.9
Proline	22.2	21.0	22.8	27.0	16.2	3.3	4.4	2.8	2.8	4.8
Tyrosine	12.3	9.0	15.0	17.7	6.3	1.8	1.8	1.8	1.8	1.9
Valine	13.5	9.3	16.2	20.1	5.7	2.0	1.9	2.0	2.1	1.7
Methionine	3.6	1.8	5.1	5.7	1.2	0.5	0.4	0.6	0.6	0.4
Cysteine	31.8	26.4	35.7	25.5	39.0	4.7	5.5	4.4	2.7	11.5
Isoleucine	8.4	5.1	10.8	11.4	4.8	1.2	1.1	1.3	1.2	1.4
Leucine	8.1	5.4	10.5	11.1	4.8	1.2	1.1	1.3	1.2	1.4
Phenylalanine	9.9	5.7	12.9	15.6	3.0	1.5	1.2	1.6	1.6	0.9
Ornithine	57.6	40.8	69.6	90.6	18.6	8.5	8.5	8.6	9.4	5.5
Tryptophan	5.7	4.5	6.3	5.4	5.7	0.8	1.0	0.8	0.6	1.7
Lysine	18.0	10.2	23.7	27.3	7.2	2.7	2.1	2.9	2.8	2.1

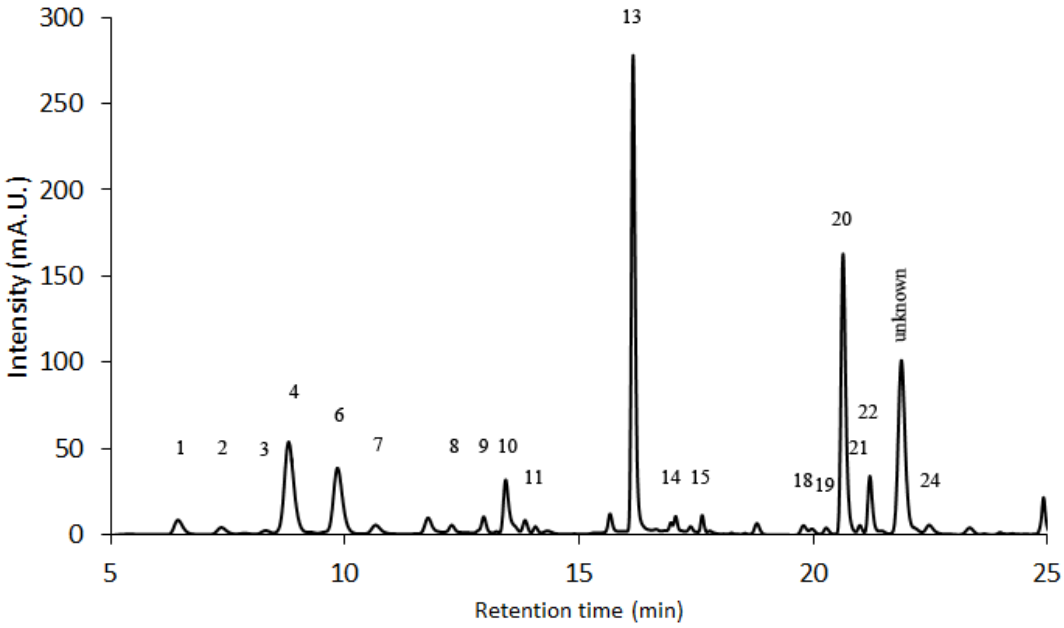


Figure 5.10 Sample chromatogram of a PITC derivatised fingermark extract. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm. For the amino acids corresponding to the numbers, refer to Table 5.4.

In this study, samples were collected from the middle 3 fingers for each donor. Before attempting to discern differences between donors, the intra-donor variation was assessed. As can be seen from Figure 5.11, there are marked differences between the absolute concentrations of amino acids within the 3 fingers of a single donor. This may be explained by the fact that with each finger, the size of the fingertip and deposition factors such as pressure and angle will be different, where some donors showed much more pronounced differences than others. Relative concentrations were calculated as a percentage by dividing the concentration of the amino acid by the sum of all amino acids' concentrations. In this case, the variation due to the intra donor effect was decreased across most analytes (Figure 5.12).

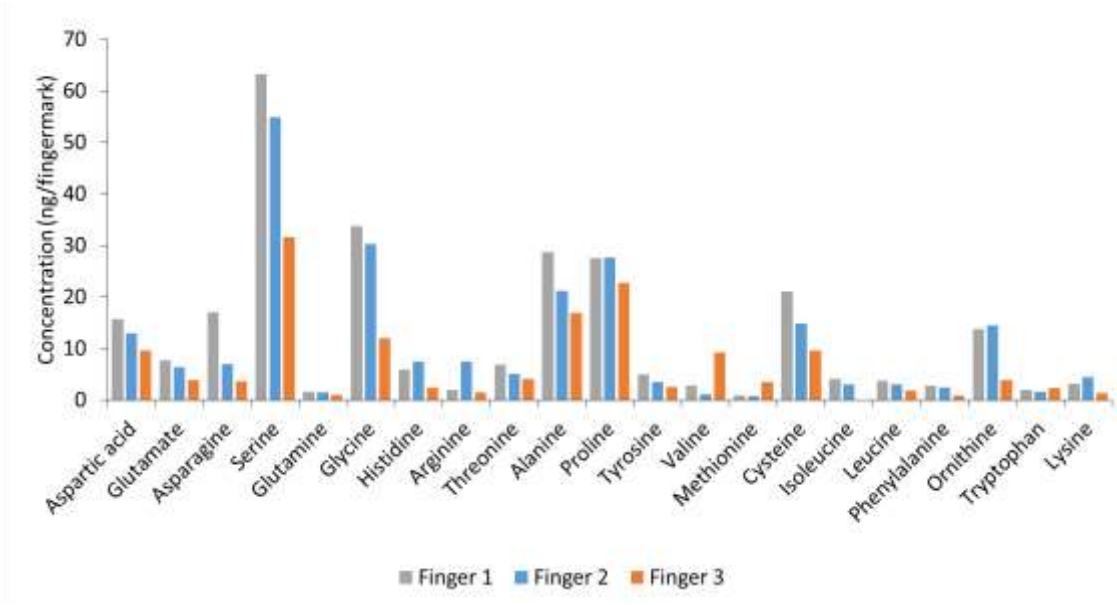


Figure 5.11 Comparison of the absolute amino acid concentrations gained from 3 different fingers of a single donor.

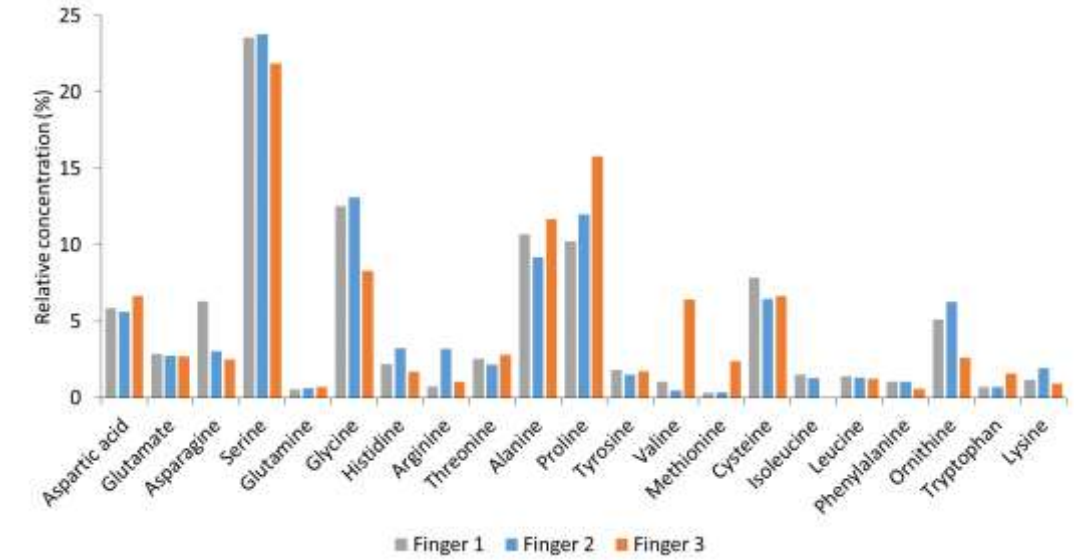


Figure 5.12 Comparison of the amino acid concentrations, relative to the sum of all amino acids' concentrations, gained from 3 different fingers of a single donor.

5.3.4.1 Donor traits

Inter-donor variation was investigated by assessing possible differences in the results due to donor traits (Table 5.9). Figure 5.13 shows that the relative amino acid abundance is very similar in both sexes. The absolute concentrations are vastly different, where male donors gave a higher concentration for every amino acid (Figure 5.14). The difference may possibly be due to the increased size of the

fingertips of most male donors, male donors applying more pressure when depositing fingermark samples or due to the difference in metabolic rate/eccrine gland activity. The report by Coltman *et al.* in 1966 indicated that there was little difference between the amino acids found in pure sweat from male and female donors [181]. De Puit *et al.* found that ornithine, lysine and histidine appeared to show the biggest differences between the sexes, whereas methionine, lysine and phenylalanine gave the largest difference in this study [35]. This difference may be due to the same sample population limitations outlined above.

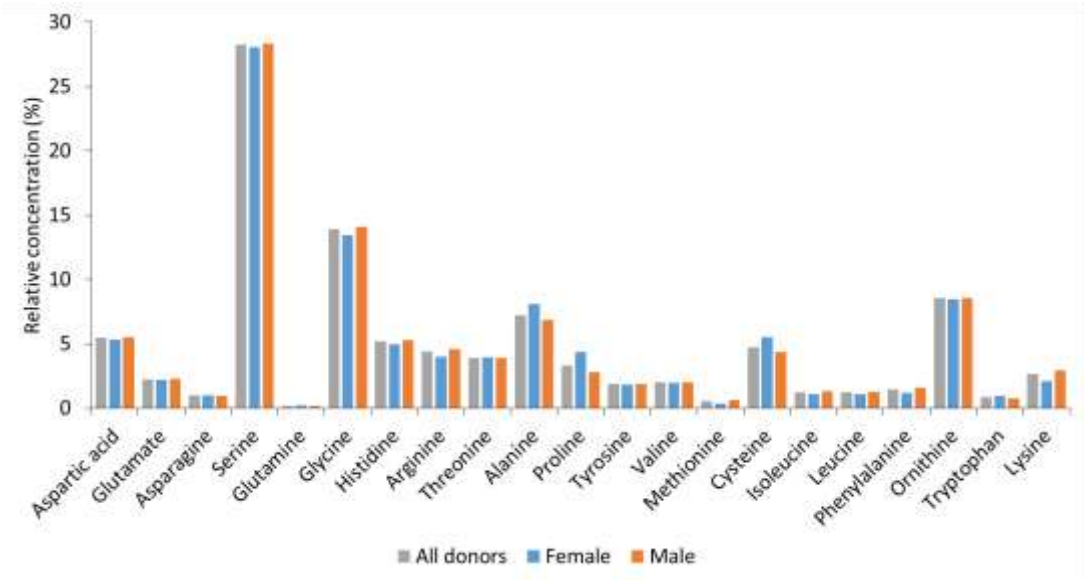


Figure 5.13 Relative, to the sum of total amino acid concentration, abundance of amino acids in all donors, males, and females.

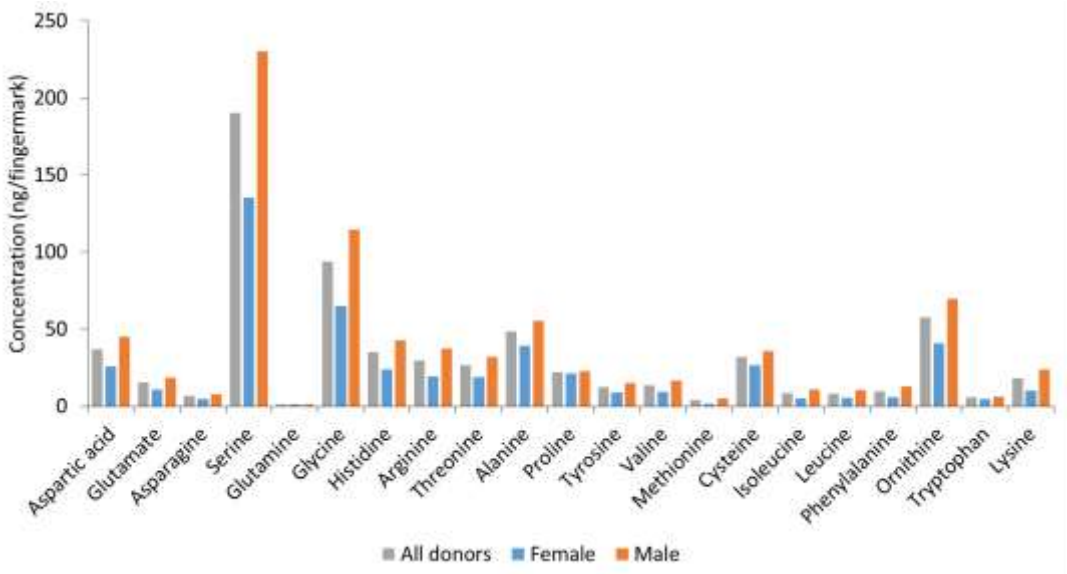


Figure 5.14 Absolute abundance of amino acids in all donors, males, and females.

The greatest differences between donors over and under the age of 25 were shown in the ornithine, histidine and phenylalanine content with both the average and absolute concentrations (Figure 5.15 and 5.16 respectively). All amino acids were found in much greater absolute concentrations in donors under the age of 25, except for cysteine and tryptophan. One possible reason for the increase in amino acid content of donors under 25 may be the rate of excretion of amino acids and/or the difference in the quantity of eccrine gland secretion. These findings can be compared to the results by Armstrong and Stave which indicate that the absolute quantities of plasma free amino acids in children and adults are very similar [222]. While Gitlitz *et al.* showed that the relationship between amino acids in sweat and plasma is dependent on the specific amino acid [223], the actual excretion of plasma amino acids may be different for young and older donors. Differences in the population and analytical approach may also be responsible for the discrepancies in the results, in addition to dealing with trace quantities of amino acids present in fingermark deposits compared to bulk sweat analysis.

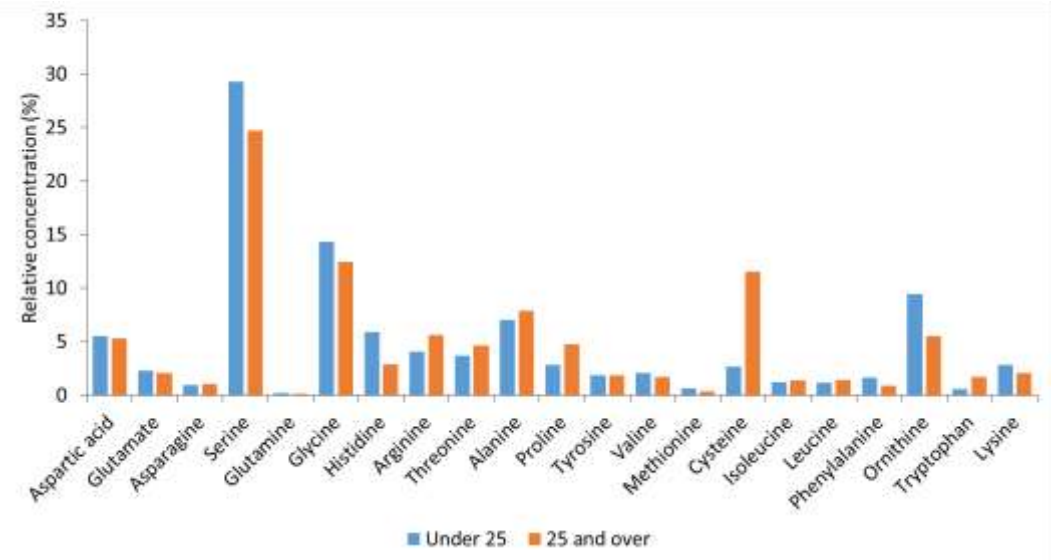


Figure 5.15 Relative, to the sum of total amino acid concentration, abundance in donors under and over the age of 25 years.

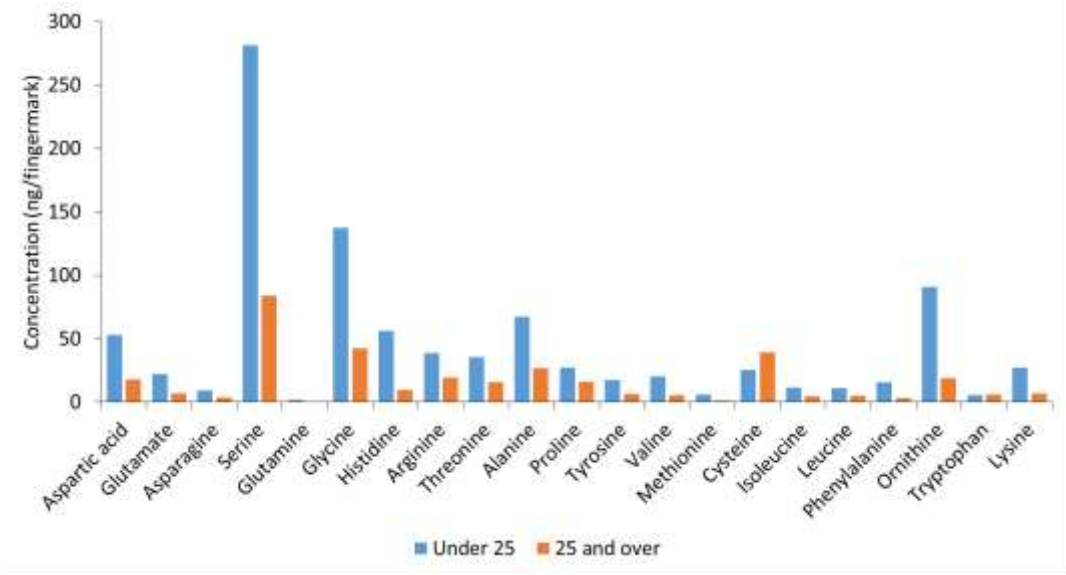


Figure 5.16 Absolute abundance of amino acids in donors under and over the age of 25 years.

5.3.4.1 Donor habits

In addition to the donor traits discussed above, the effect of donor habits on the amino acid content were examined (Table 5.10). The major differences between donors that had washed their hands to those that had not within the hour prior to fingermark deposition are in the relative amounts of glutamine, proline and cysteine (Figure 5.17). Although the relative profile of amino acids appeared visually similar, the absolute concentration profile showed that there was a marked difference in nearly all amino acids (Figure 5.18). Donors who had washed their hands had a much lower concentration of amino acids (except for glutamine), as to be expected from the water soluble nature of these analytes. These results mirror the conclusion drawn in Chapter 4, where 1,2-indanedione/zinc chloride gave a weaker response to deposits from donors who had washed their hands.

Table 5.10 Abundance of amino acids, both absolute values and relative to the sum of total amino acid concentration, of donor habits.

Amino acid	Absolute concentration (ng/fingermark)						Relative concentration (%)					
	Washed hands	Unwashed hands	Food consumed	No food consumed	Cosmetics used (<12 hrs)	No cosmetics used (<12 hrs)	Washed hands	Unwashed hands	Food consumed	No food consumed	Cosmetics used (<12 hrs)	No cosmetics used (<12 hrs)
Aspartic acid	21.9	40.5	39.9	35.4	25.8	42.0	6.2	5.4	5.8	5.3	5.8	5.4
Glutamate	8.7	16.8	16.2	14.7	10.2	17.4	2.4	2.2	2.4	2.2	2.3	2.2
Asparagine	4.2	7.2	7.5	6.0	4.2	7.5	1.2	0.9	1.1	0.9	0.9	1.0
Serine	91.5	215.1	197.1	187.2	117.3	224.7	25.8	28.5	28.8	28.0	26.3	28.7
Glutamine	1.5	1.2	2.1	0.6	1.2	1.2	0.4	0.1	0.3	0.1	0.3	0.1
Glycine	45.0	105.9	93.9	93.6	56.4	111.3	12.7	14.0	13.7	14.0	12.7	14.2
Histidine	16.2	39.6	34.5	35.1	16.2	43.5	4.6	5.2	5.0	5.2	3.7	5.6
Arginine	18.3	32.7	28.5	30.3	19.8	34.5	5.1	4.3	4.2	4.5	4.4	4.4
Threonine	16.5	28.8	24.9	27.0	17.7	30.3	4.7	3.8	3.6	4.0	4.0	3.9
Alanine	31.2	52.8	49.2	48.3	34.2	55.2	8.8	7.0	7.2	7.2	7.7	7.1
Proline	17.7	23.1	21.6	22.5	18.6	23.7	5.0	3.1	3.1	3.3	4.2	3.0
Tyrosine	6.3	14.1	12.3	12.6	8.4	14.4	1.8	1.9	1.8	1.9	1.9	1.8
Valine	6.9	15.0	13.5	13.5	8.4	15.9	1.9	2.0	2.0	2.0	1.9	2.0
Methionine	1.5	4.2	3.0	3.9	1.8	4.5	0.4	0.6	0.5	0.6	0.4	0.6
Cysteine	12.9	36.6	28.5	33.3	39.6	28.2	3.6	4.8	4.2	5.0	8.9	3.6
Isoleucine	3.6	9.6	8.4	8.4	6.6	9.3	1.0	1.3	1.2	1.2	1.5	1.2
Leucine	4.2	9.3	8.4	8.1	6.0	9.3	1.1	1.2	1.2	1.2	1.3	1.2
Phenylalanine	5.4	10.8	9.9	9.6	5.4	11.7	1.5	1.4	1.5	1.5	1.2	1.5
Ornithine	30.3	64.5	63.6	54.6	32.7	69.3	8.5	8.5	9.3	8.2	7.3	8.9
Tryptophan	3.3	6.3	5.1	5.7	5.1	6.0	0.9	0.8	0.8	0.9	1.1	0.8
Lysine	7.5	20.7	17.1	18.3	9.9	21.9	2.1	2.7	2.5	2.8	2.2	2.8

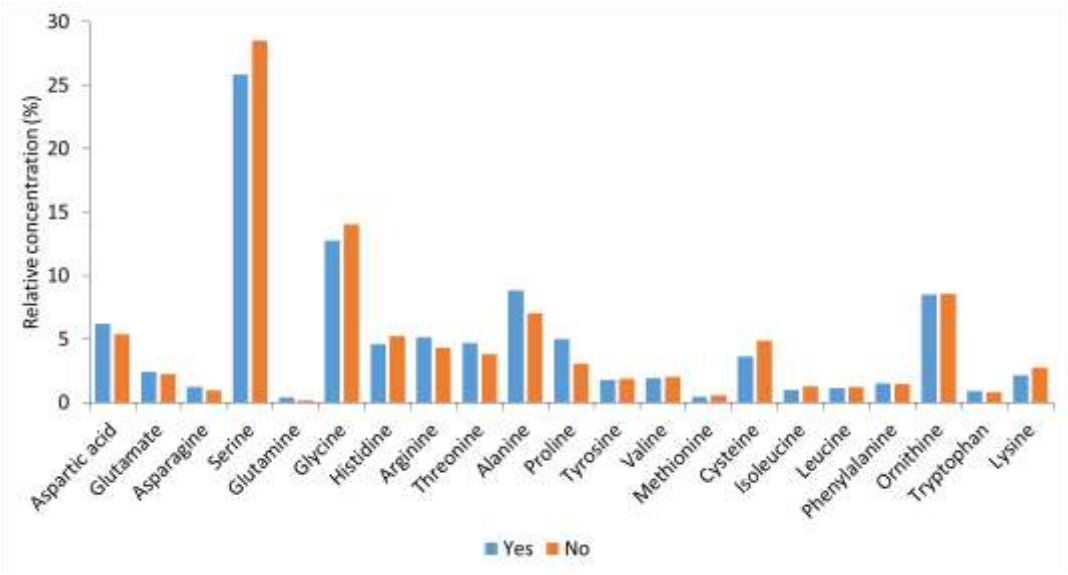


Figure 5.17 Relative, to the sum of total amino acid concentration, abundance in donors who had and had not washed their hands within one hour of fingermark deposition.

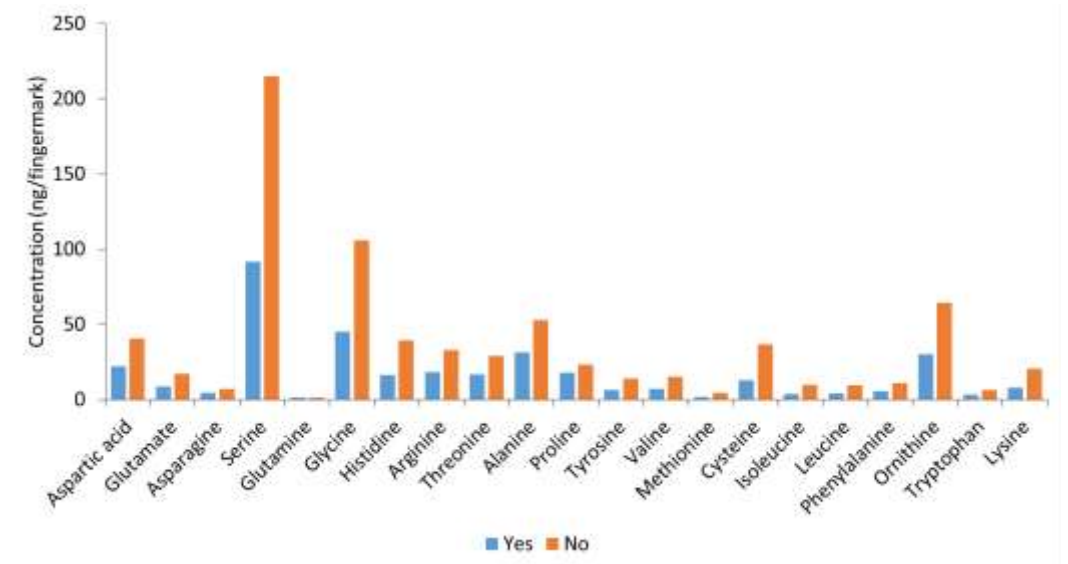


Figure 5.18 Absolute abundance of amino acids in donors who had and had not washed their hands within one hour of fingermark deposition.

Glutamine, methionine and cysteine were the three amino acids that showed the most significant difference in both their relative and absolute concentrations between donors who had and had not recently consumed food, according to Table 5.10. On closer inspection of Figure 5.19 and Figure 5.20, the differences between each of these three amino acid appeared to minimal. Especially glutamine and methionine are also of very low concentration, where the difference may not be statistically significant. All other amino acids displayed similar profiles whether the donors had or had not consumed food.

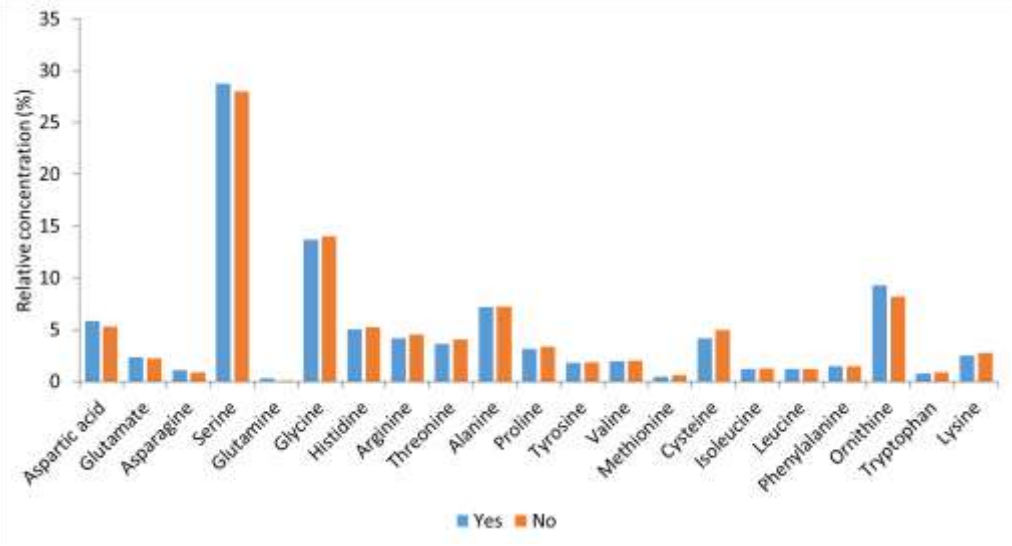


Figure 5.19 Relative, to the sum of total amino acid concentration, abundance of amino acids in donors who had or had not consumed food within one hour prior to fingermark deposition.

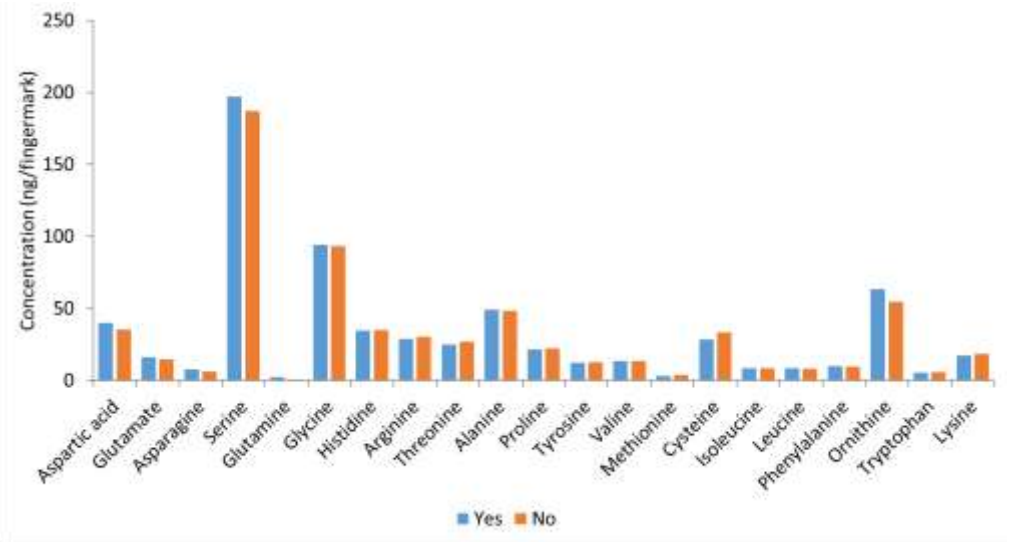


Figure 5.20 Absolute abundance of amino acids in donors who had or had not consumed food within one hour prior to fingermark deposition.

Serine, glycine, ornithine and histidine displayed the largest difference between donors who had used cosmetics within 12 hours prior to giving fingermark deposits and those who had not (Figure 5.21 and 5.22). These changes held true for both absolute and relative concentrations of all amino acids. Cosmetics can contain any number of amino acids, therefore the specific product used by each donor would need to be known to discern whether the amino acid present in the cosmetic product has an effect on the qualitative and quantitative profile of amino acids present in fingermark deposits. More detailed investigations into this aspect were outside the scope of this project but may be of interest in any subsequent studies.

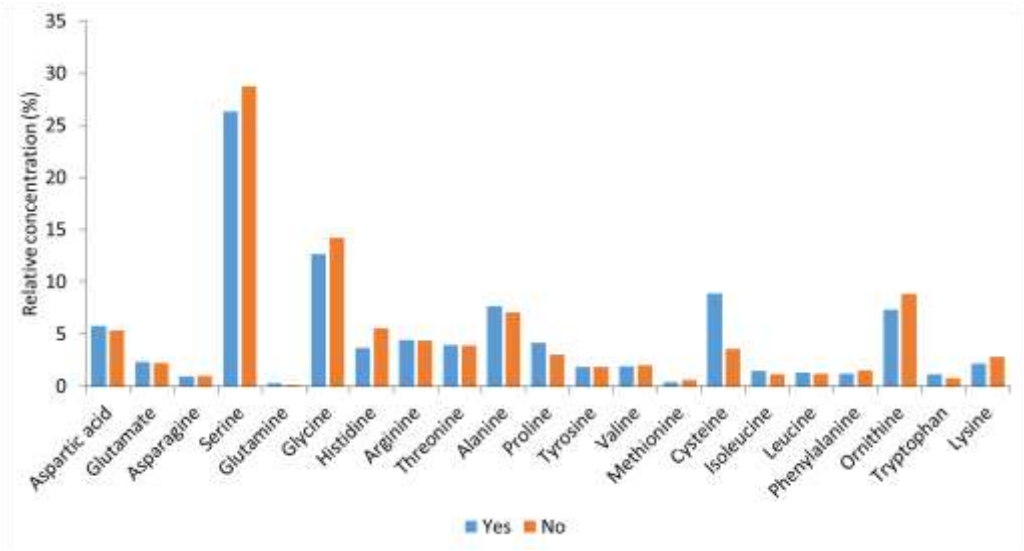


Figure 5.21 Relative abundance of amino acids, relative to the sum of total amino acid concentration, for donors who had and had not used cosmetics within 12 hours of giving fingermark deposits.

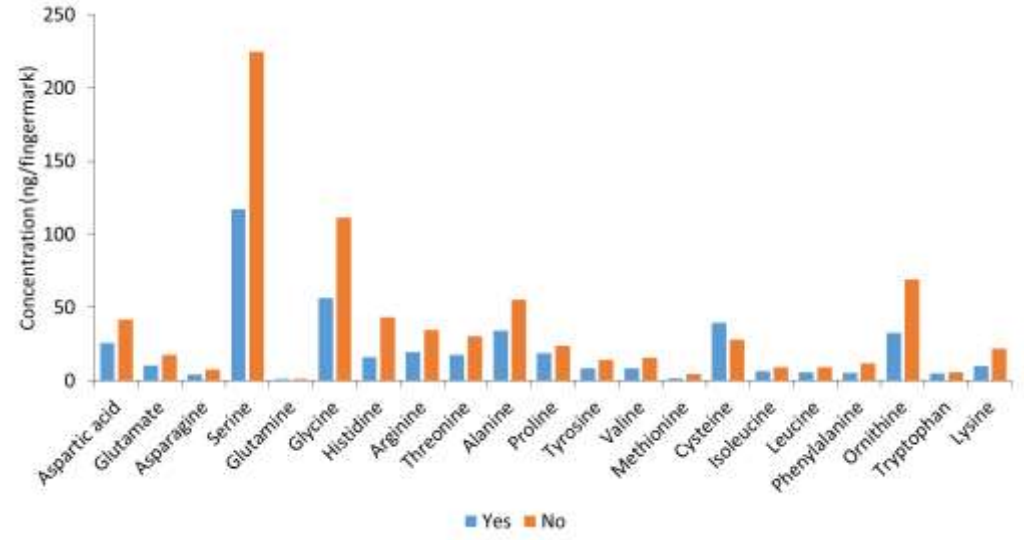


Figure 5.22 Absolute abundance of amino acids for donors who had and had not used cosmetics within 12 hours of giving fingermark deposits.

5.3.5 Statistical data evaluation

There are several approaches to statistically evaluating the data obtained above. A multivariate chemometric approach can use the entire chromatogram to discern any possible differences between donors [224]. PCA can provide clustering of the samples according to its discriminating variables. Reduction of complex data is achieved by combining related variables and thereby reducing the number of dimensions, allowing relationships within the samples to be discerned much more readily [195]. While this is potentially the most descriptive method, as it is

influenced by all data points, it requires rigorous data pre-processing such as fixing the retention times for the analytes of interest, normalisation and baseline corrections [224]. Alternatively, chemometric analysis can also be applied to the absolute and relative concentrations of the analytes. While this approach is less powerful than the method described above, the data pre-processing in this instance is potentially much simpler as only the analytes of interest are considered for the statistical model. A further, simpler approach used comprehensively below is the application of more conventional statistics by assessing the absolute and relative concentrations of the analytes, as discussed above [194, 225-227].

As in Chapter 4, the Mann-Whitney U test was used to compare the medians of the absolute and relative concentrations of all samples to determine whether there are significant differences due to donor traits and habits (Table 5.1). While the relative abundance can reduce the effect of the deposition factors, important information may be lost by ignoring the absolute amounts. The average concentration for the three fingers of each donor was used to simplify the required statistics and to remove any intra-donor variation. While the probability and Z score values were calculated for all amino acids, only the 5 most abundant (serine, glycine, ornithine, alanine, and aspartic acid) are discussed below. These were found in all donors, make up about 63 % of the total amino acid content and could be more reliably detected due to their higher concentrations.

5.3.5.1 Donor traits

The sex of the donor did not appear to affect the most abundant amino acids, where an average p value of 0.431 and a Z score of -0.847 was calculated for the relative abundance, and $p = 0.170$ and $Z = -1.394$ for the absolute concentration (Table 5.11). As can be seen from Figure 5.15, although the male donors appeared to deposit larger quantities of amino acids, this was not deemed statistically significant. In the pilot and donor study in Chapter 4, it was also found that biological sex did not appear to affect the grade given to developed fingermark samples and the results above confirm those findings. Out of 21 amino acids, only leucine, methionine, asparagine and glutamic acid were shown to be significantly different in the absolute concentrations. For relative concentration comparisons, asparagine showed no

significant difference but proline and isoleucine did. Isoleucine and especially leucine (which also had the lowest p value) are key amino acids that promote muscle growth during prolonged exercise [228]. As males have more muscle mass on average than females [229], this may explain why these amino acids were twice as abundant in male donors (Table 5.9). This effect has also been observed in previous studies of the plasma amino acid concentrations of males and females [52, 222].

There was a significant difference (average p = 0.025, Z = -2.291) between donors over and under the age of 25 in the absolute concentration of the 5 most abundant amino acids, see Table 5.11. The Z score is much larger than the critical value (Z = -1.96), meaning that the null hypothesis (i.e. that no significant difference exists) can be rejected with greater than 98.6 % confidence. These findings are again in agreement with the results of the pilot and donor study, further enforcing that differences in the amino acid content may exist as a function of donor age. When comparing the relative concentrations, the average p and Z values were 0.074 and -2.151, respectively. This means that there is no significant difference between donors over and under the age of 25. However, when alanine is taken out the calculations, the age is a statistically significant effect for the other 4 amino acids (average p = 0.023, Z = -2.419). In addition to the most abundant amino acids, the absolute concentrations of histidine, valine, tyrosine, lysine, phenylalanine, glutamic acid and glutamine were also significantly different as a function of the age of the donor. If the age groups are changed to show those donors under 15 and those 20 or over, the effect is even more pronounced and all amino acids except for leucine, isoleucine, tryptophan and cysteine were found to be significantly different.

Table 5.11 Statistical values gained from Mann-Whitney U tests, with the relative and absolute concentrations of amino acids given as a function of the independent variables.

Variable	Absolute concentration				Relative concentration			
	Sex p value	**Sex Z value**	**Age p value**	**Age Z value**	**Sex p value**	**Sex Z value**	**Age p value**	**Age Z value**
Aspartic acid	0.105	-1.622	0.023	-2.268	0.198	-1.287	0.031	-2.151
Glutamate	0.046	-1.995	0.029	-2.190	0.031	-2.152	0.078	-1.762
Asparagine	0.031	-2.153	0.182	-1.333	0.160	-1.406	0.977	-0.029
Serine	0.172	-1.366	0.035	-2.112	0.836	-0.206	0.015	-2.443
Glutamine	0.673	-0.422	0.050	-1.961	0.721	-0.357	0.160	-1.404
Glycine	0.138	-1.484	0.038	-2.073	0.510	-0.658	0.042	-2.034
Histidine	0.267	-1.111	0.008	-2.657	0.761	-0.305	0.003	-2.988
Arginine	0.082	-1.740	0.147	-1.450	0.275	-1.091	0.961	-0.049
Threonine	0.178	-1.346	0.153	-1.431	0.746	-0.324	0.255	-1.139
Alanine	0.267	-1.111	0.020	-2.326	0.219	-1.229	0.280	-1.080
Proline	0.746	-0.324	0.136	-1.489	0.009	-2.624	0.676	-0.419
Tyrosine	0.089	-1.700	0.010	-2.560	0.992	-0.010	0.465	-0.730
Valine	0.171	-1.370	0.009	-2.606	0.370	-0.897	0.099	-1.650
Methionine	0.025	-2.242	0.052	-1.944	0.047	-1.983	0.158	-1.411
Cysteine	0.116	-1.573	0.620	-0.496	0.665	-0.432	0.003	-2.988
Isoleucine	0.058	-1.895	0.545	-0.606	0.040	-2.055	0.356	-0.923
Leucine	0.018	-2.369	0.239	-1.178	0.007	-2.703	0.017	-2.384
Phenylalanine	0.101	-1.641	0.014	-2.462	0.118	-1.563	0.002	-3.144
Ornithine	0.166	-1.386	0.007	-2.676	0.393	-0.855	0.002	-3.046
Tryptophan	0.761	-0.305	0.454	-0.749	0.205	-1.268	0.026	-2.229
Lysine	0.089	-1.7	0.012	-2.501	0.143	-1.464	0.009	-2.618

5.3.5.2 Donor habits

Donors who had or had not washed their hands prior to fingermark deposition were found not to give significantly different deposits as a function of their absolute and relative amino acid concentrations ($p = 0.161$, $Z = -1.494$ and $p = 0.240$, $Z = -1.489$, respectively). Although this is different to the results from the pilot and donor study, in this preliminary study only 10 out of 50 donors had washed their hands prior to sample collection. Using a larger set of donors may change these results to a significant effect, as serine, glycine and alanine are already near the critical value for the current population.

No statistically significant dissimilarity was found between the absolute amino acid concentration from donors who had or had not recently consumed food ($p = 0.920$, $Z = -0.100$) or had applied cosmetics ($p = 0.305$, $Z = -1.052$), see Table 5.12. This was in agreement with the relative abundance (food: $p = 0.885$, $Z = -0.146$; cosmetics: $p = 0.464$, $Z = -0.836$) and the results of the donor study in Chapter 4. Figure 5.22, depicting the absolute concentrations of amino acids in donors who had and had not used cosmetics, would suggest that there is a marked difference in most amino acids between the two donor groups. It is only when the standard deviations of the amino acids are taken into account, that the reason becomes evident. For example, the standard deviation of donors who had not used cosmetic products was 405 ng/fingermark for serine and 137 ng/fingermark for donors who had used cosmetic products. It is likely due to this large intra-variable deviation that the Wilcoxon sign rank test did not find a statistically significant difference due to cosmetic use, despite the visual examination of the figures suggesting otherwise.

The absolute and relative abundances of the amino acids found in latent fingermark deposits were further investigated using a chemometric approach. PCA was applied to all donor variables, where it was found that no clustering occurred due to any trait or habit. Even when the number of amino acids was limited to those that showed a significant difference in the Mann-Whitney U tests, no clustering was observed. This may be an effect of the large standard deviation associated with each variable and amino acid, due to the large intra- and inter-donor variation.

Table 5.12 Statistical values gained from Mann-Whitney U tests, with the relative and absolute concentrations of amino acids given as an effect of the independent variables.

Variable	Absolute concentration						Relative concentration					
	Washing of hands p value	Washing of hands Z value	Food p value	Food Z value	Cosmetics p value	Cosmetics Z value	Washing of hands p value	Washing of hands Z value	Food p value	Food Z value	Cosmetics p value	Cosmetics Z value
Aspartic acid	0.182	-1.334	0.917	-0.104	0.270	-1.102	0.320	-0.994	0.739	-0.333	0.279	-1.081
Glutamate	0.099	-1.649	0.884	-0.146	0.339	-0.957	0.771	-0.291	0.533	-0.624	0.114	-1.581
Asparagine	0.207	-1.261	0.708	-0.374	0.328	-0.978	0.961	-0.049	0.819	-0.229	0.092	-1.685
Serine	0.049	-1.965	0.884	-0.146	0.417	-0.811	0.059	-1.892	1.000	0.000	0.835	-0.208
Glutamine	0.979	-0.027	0.013	-2.474	0.731	-0.344	0.873	-0.160	0.019	-2.336	0.663	-0.435
Glycine	0.059	-1.892	0.934	-0.083	0.382	-0.873	0.094	-1.673	0.819	-0.229	0.349	-0.936
Histidine	0.121	-1.552	0.771	-0.291	0.064	-1.851	0.344	-0.946	0.819	-0.229	0.011	-2.538
Arginine	0.199	-1.285	0.519	-0.645	0.176	-1.352	0.942	-0.073	0.493	-0.686	0.157	-1.414
Threonine	0.160	-1.407	0.868	-0.166	0.253	-1.144	0.846	-0.194	0.868	-0.166	0.755	-0.312
Alanine	0.308	-1.019	0.884	-0.146	0.318	-0.998	0.012	-2.522	0.950	-0.062	0.755	-0.312
Proline	0.790	-0.267	0.934	-0.083	0.819	-0.229	0.039	-2.062	0.934	-0.083	0.852	-0.187
Tyrosine	0.065	-1.843	0.739	-0.333	0.442	-0.769	0.903	-0.121	0.739	-0.333	0.755	-0.312
Valine	0.158	-1.411	0.851	-0.188	0.260	-1.126	0.253	-1.143	0.359	-0.918	0.802	-0.250
Methionine	0.228	-1.205	0.704	-0.379	0.238	-1.181	0.338	-0.959	0.883	-0.148	0.238	-1.181
Cysteine	0.007	-2.692	0.526	-0.634	0.493	-0.686	0.190	-1.310	0.333	-0.967	0.077	-1.768
Isoleucine	0.088	-1.707	0.932	-0.085	0.611	-0.509	0.051	-1.955	0.524	-0.637	0.227	-1.209
Leucine	0.017	-2.377	0.934	-0.083	0.835	-0.208	0.265	-1.116	0.755	-0.312	0.868	-0.166
Phenylalanine	0.094	-1.673	0.787	-0.270	0.220	-1.227	0.225	-1.213	0.339	-0.957	0.070	-1.809
Ornithine	0.207	-1.261	0.983	-0.021	0.140	-1.477	0.716	-0.364	0.917	-0.104	0.100	-1.643
Tryptophan	0.047	-1.989	0.405	-0.832	0.677	-0.416	0.344	-0.946	0.603	-0.520	0.197	-1.289
Lysine	0.160	-1.407	0.884	-0.146	0.067	-1.830	0.662	-0.437	0.724	-0.354	0.021	-2.308

5.3.6 Simulants

As demonstrated above, real fingermarks from a range of donors show a large variation in amino acid content. This presents challenges when attempting to develop and validate fingermark treatment options as the donor(s) can have a large impact on the efficacy of a method. Fingermark simulant samples have been proposed as one possible method of making a standardised fingermark, and it is therefore important that their reaction with development reagents closely mimics real samples [100]. As previously reported by Zadnik *et al.*, and discussed in Chapter 1, this is not always the case, suggesting that the chemical composition may be different [151]. Figure 5.23 demonstrates the chemical difference between the extraction of a real fingermark exhibit and that of a commercially sourced amino acid simulant sample which was stamped onto the filter paper substrate. The chromatogram of the simulant is dominated by a peak corresponding to cysteine (18.39 min) and unknown peaks at 5.85 min and 19.39 min. In addition, peaks corresponding to alanine and ornithine were identified. The relative (and absolute) concentrations, especially for cysteine, are vastly different between the two chromatograms. While simulants can make an appealing case through their ease of use and reproducibility, these results highlight the need for caution when attempting to use simulant samples as realistic fingermark replacements.

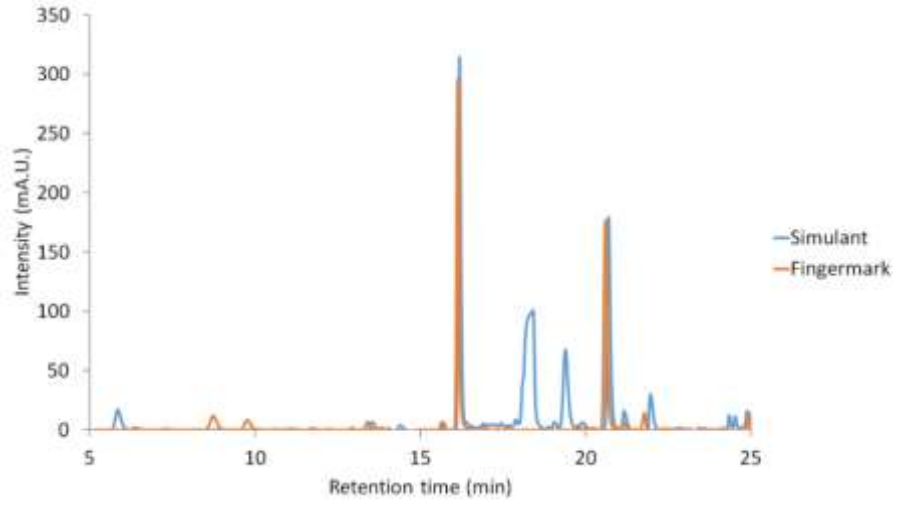


Figure 5.23 Chromatogram comparison of PITC derivatised analytes and IS solutions of a real fingermark exhibit versus an amino acid simulant sample. Amino acids were analysed using an Agilent XDB C18 column (150 mm x 4.6 mm I.D, 5 µm particle size and a diode array detector set at 254 nm.

5.4 Conclusions

A simplified method for the analysis of free amino acids was developed from Gheshlaghi *et al.*'s work to offer separation of 21 amino acids and 2 internal standards [80]. This method was adapted to suit the extraction and separation of amino acids from latent fingermark deposits on porous substrates and is amenable to large scale studies. Average extraction efficiencies of 83 % and correlation coefficients of 0.993 for the calibration curves were achieved with a limit of detection of 0.96 pg/µL.

Fingermark samples from 50 donors were analysed, where exhibits from 3 fingers for each donor were collected. It was found that serine was the most abundant amino acid in all samples, followed by glycine, ornithine, alanine, and aspartic acid (in that order). The average concentration of each amino acid per fingermark was found to be between 1.2 ng (glutamine) and 190.2 ng (serine). The sum of the average amino acid concentrations was shown to be 675 ng per fingermark, but ranged from 40 to 8050 ng per fingermark. The results suggest that insignificant intra-donor variation exists within the relative amino acid concentrations of the three fingers, except within tryptophan.

The inter-donor studies can be split into those which concern the donor traits (i.e. age and sex) and those that look at donor habits (i.e. recent food consumption and washing of hands). Comparing the absolute and relative concentrations of the 5 most abundant amino acids (serine, glycine, ornithine, alanine, and aspartic acid), it was found that there was no significant difference due the food consumption, use of cosmetics, washing of hands or biological sex. The absolute concentration deposited by donors over and under the age of 25 was statistically dissimilar in all 5 amino acids, and different for all relative amino acid concentrations, apart from alanine, as well. Aside from the results for donors who had and had not washed their hands, all outcomes reflect the findings of the donor study. Larger sample populations may result in total agreement between the studies, as only 10 out of 50 donors had washed their hands.

Chemometric analyses, in the form of PCA, of the data showed that no significant clustering occurred for any donor trait or habit. Using the results of the Mann-Whitney U tests, the number of amino acids were limited to those that were found to be significantly different between variables; however, no further clustering was observed.

Comparisons of amino acid simulant samples show significant differences to those of real latent fingermark deposits, reinforcing the findings by Zadnik *et al.* that the chemical composition of simulants is likely to be different to real fingermarks [151].

Chapter 6

Liquid Chromatography – Mass Spectrometry analysis of amino acid content of latent fingermark deposits

6.1 Introduction

In addition to the analysis of amino acids present in latent fingermarks using high performance liquid chromatography (HPLC) coupled to an ultraviolet - diode array detector (UV-DAD), HPLC systems coupled to mass spectrometry detectors (LC-MS) were used for the same samples. Compared to more conventional HPLC-UV detection systems, LC-MS is a more specific analytical technique, monitoring the *m/z* ratio of parent ions and fragments, thereby often reducing the need for complete separation [64]. In addition, MS analytical techniques generally offer a much better sensitivity (i.e. limit of detection) over UV-DAD for the same analytes [64].

The two main components of MS systems are the ion source and the mass analyser. Two complementary, and most commonly used, ionisation sources are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) [63]. As with the thermospray technique, these are both classed as "soft ionisation" methods causing little in-source fragmentation, leaving the majority of analytes in their molecular ion form [63]. Both ESI and APCI ionise analytes at atmospheric pressure, but differ in the actual ionisation method [63]. ESI has been found invaluable, especially in a clinical context, due to its capabilities of charging a large range of analyte sizes, polarities and concentrations in complex biological matrices with high sensitivity, reliability and robustness [230, 231].

For ESI equipped mass spectrometers, quadrupole, ion traps and Orbitrap techniques are three examples of mass analysers that may be used. These separate the analytes according to their *m/z* ratio and release them to the detector for data collection. The quadrupole mass analyser, first coupled to GC in the 1950s, works by using electric fields, typically emitted from 4 identical metal rods, which force the resonant ions to spiral towards the detector. In a triple quadrupole (QqQ) system, 2 quadrupoles and a collision cell can also be used in tandem to give MS/MS (i.e. fragmentation) capabilities. The combination of 3 quadrupoles offers a range of mass spectrometry acquisition modes, including MS Scan, single ion monitoring (SIM), product and precursor ion scans, selected reaction monitoring (SRM), multiple reaction monitoring (MRM) and MS scan surveys. Triple quadrupoles are typically used for routine quantitation work, due to the selectivity and sensitivity of such mass

analysers when operated in SRM or MRM mode [232]. Quadrupole ion traps use constant direct current and oscillating electric fields to trap ions. Similarly to QqQ, data can be acquired in MS Scan, SIM and SRM mode, but ion traps are most suited to conduct multiple stage fragmentation experiments (MS^n) for the determination of unknowns and impurities in samples [233]. However, both the triple quadrupole and quadrupole ion trap systems are generally not suited for high precision work, as they have low mass accuracy (~50 ppm) and are commonly operated at unit mass resolution [233]. Orbitrap mass analysers are a new form of high precision ion traps, firstly described in 2000 by Makarov [234]. Orbitraps are usually used for "discovery" work, where high mass resolution, high mass accuracy and multiple fragmentation stages aid the identification of unknown analytes in the samples [233]. In combination with quadrupole ion traps (low resolution MS^n data), Orbitraps can provide high resolution (e.g., 400 000) and high mass resolution (< 1 ppm, with external calibration) MS data.

LC-MS was an important development for the qualitative and quantitative detection of analytes which were hydrophilic, thermally labile, of high molecular weight or non-volatile, and therefore not amenable to GC-MS analysis [235, 236]. LC-MS has now found a widespread use in a number of applications, including in the biomedical, environmental and food industries [237].

6.1.1 LC-MS application in forensic science

Although GC-MS is still widely used in many analytical tests, LC-MS has now also "become a reliable and robust analytical technique for routine analysis" for polarisable analytes [235]. It is now seen as a vital technique in the forensic sciences for samples that are less suited to GC-MS analysis. A number of recent reviews detail the applications of LC-MS in the forensic sciences, where the abundance of research has been performed in the toxicology discipline and analytes include drugs (e.g. illicit and prescription), metabolites and poisons in a variety of biological matrices [235, 236, 238]. However, LC-MS has also found use in trace analysis for the detection of chemical warfare agents, explosives and dyes in a forensic context [236]. In addition to these tasks, LC-MS with automated online sample preparation,

such as solid-phase extraction, can provide quick, sensitive and reliable results for high-throughput laboratories [233, 236].

As an example, LC is amenable to the separation of explosive residues from other interferences, which are often difficult to analyse due to their chemical instability and polarity. The identification and profiling of explosive matter from postblast debris is of forensic importance at bombing scenes, where a bomb's chemical composition may reveal the type of explosive device that was used [233]. Recently, Sanchez *et al.* developed an LC-APCI-MS method in the negative ion mode for explosive residue detection [239]. Good reproducibility and fast analysis time (<30 min) with detection limits in the femtogram-per-litre range were achieved.

A further example of the importance of LC-MS for forensic science is the application of high resolution LC-Time of flight-MS in drug analysis. Databases for known drugs can be constructed from a compound's elemental composition, which is useful for the matching and subsequent identification of unknown substances. This is particularly useful for the monitoring of new drugs and their potential metabolites. The work by Guale *et al.* indicated that a quick (13 minute analysis) screening method could differentiate between a number of forensically relevant prescription and illicit drugs in both urine and blood [240]. As such, they were able to provide "scientific evidence of the use of a drug, its origins, manner of absorption and relative amounts" using a method that is useable for routine analysis [240].

6.1.2 Analysis of amino acids using LC-MS

LC-MS is becoming increasingly important for research performed on amino acids in a variety of matrices, including biological specimens, plant material and food products [241-247]. Traditionally, this body of research was predominantly performed on HPLC UV-Vis systems; however, due to the advantages described above, LC-MS is becoming increasingly popular [242, 243].

As with most analytical techniques, additional sample preparation is to be avoided where possible, and unlike HPLC UV-Vis, derivatisation may not be necessary for analyte detection when using MS [243]. Sample derivatisation can result in potential side reactions, be subject to derivative instability, as well as possible reagent

interferences and the longer sample preparation associated with it [248]. Van Leuken *et al.* were the first group to separate underivatised amino acids using LC-MS in 1993, where volatile ion-pairing agents and a triethylamine buffer system were used in the mobile phase [249]. Chaimbault *et al.* were the first to use ESI to determine the 20 proteinogenic amino acids in their underivatised form [250].

To date, the only report investigating the amino acid content in latent fingermarks using LC-MS from a forensic point of view was a study conducted by de Puit *et al.* in 2013 [35]. Nineteen amino acids were successfully detected in samples collected from 20 donors, where limits of detection were found to be between 0.1-0.2 pmol/µL. Certain variation between male and female donors was found to exist in the amino acid profile; however, due to the limited sample size this could not be reliably established [35]. The retention times of these derivatised amino acids ranged from 19 to 46 minutes, where the total run time was 67 minutes. The correlation coefficient for the calibration curve exceeded 0.95 for all amino acids, and exceeded 0.99 for 15. This method was therefore shown to be sensitive and fit for purpose, yet the sampling technique could be simplified. In addition, all samples had to be derivatised prior to injection which further complicated the sample preparation and should be avoided where possible as discussed above.

6.1.3 Aims

This chapter outlines the analysis of 20 amino acids in latent fingermarks using LC-MS coupled to an electrospray ionisation source operated in positive ion mode. Two different mass spectrometers were used (triple quadrupole and Orbitrap). As described above, the analysis of amino acids using LC-MS has been well established [241-247]. However, due to the large population size required for meaningful statistics, the overall analytical procedure needed to be simpler, quicker and suitable for amino acid analysis of fingermark residue extracted from a porous substrate. The analytical methods on both instruments were developed, optimised and validated to give a quick and efficient method for the collection and extraction of fingermark samples on a porous substrate. Studies concerning instrumental linearity, limits of detection (LODs), peak identification criteria (retention time), accuracy, precision,

lower limit of quantitation (LLOQ), in-house reproducibility, and matrix effects were undertaken to validate the analytical procedure.

6.2 Materials and method

6.2.1 Chemicals

Formic acid (99 % purity, Ajax FineChem, Australia) and methanol (Mallinckrodt, USA) were used as received and of analytical reagent grade. The surrogate standards, [^{2}H3] alanine (alanine-d_3), [^{2}H3] leucine (leucine-d_3), and [^{2}H3] glutamic acid (glutamic-d_3 acid), were purchased from CDN Isotopes (Quebec, Canada, distributed by SciVac, Hornsby, Australia); [^{2}H2] glycine (glycine-d_2) and [^{2}H5] phenyl [^{2}H3] alanine (phenyl-d_5-alanine-d_3) were purchased from Sigma Aldrich (New South Wales, Australia). For a description of all other chemicals see Chapter 5.

6.2.2 Sample preparation

Amino acid standards were prepared in water, except for l-aspartic acid which was dissolved in 50 % (v:v) methanol:water; and l-tyrosine which was dissolved in 0.1 M hydrochloric acid in 30 % (v:v) methanol:water. All amino acids were made up at a concentration of 1 g/L individually. These were then used to prepare a stock solution mixture containing 21 amino acids, each at a concentration of 40 ng/µL. These standards were used for all method development and validation purposes. The internal standard was prepared to a concentration of 40 ng/µL with deuterated alanine, glycine, glutamic acid, leucine and phenylalanine in 30 % (v:v) methanol:water. All solutions were kept refrigerated at 4 °C to prevent degradation. For all method development, simplification and validation experiments, the concentration of each amino acid and IS was 0.8 ng/µL, unless otherwise specified.

The collection of latent fingermark samples is described in Chapter 5. Samples were extracted from the filter paper by soaking in 50 % (v:v) methanol:water for one hour. 300 µL extracts were transferred into 2 mL screw top chromatography vials and 10 µL of the internal standard mixture was added. No further sample preparation was performed.

For method development experiments, extractions using 30 or 50 % (v:v) methanol:water compositions were trialled. For each solvent composition, samples were also either extracted without (30 or 60 minutes) or with agitation (5 or 10 minutes) using an ultrasonic bath (8891 Sonicator, Coler-Parmer, USA). Injection volumes of 2.5, 10, 25, and 50 µL were trialled to improve the sensitivity. The matrix effect was tested by analysing, in duplicate, 10 µL aliquots of the 40 ng/µL amino acid and IS stock solutions, spotted directly into 2 mL vials, and onto filter papers where they were extracted using either a 50 % (v:v) methanol:water soak for 1 hour, or by sonication with 50 % (v:v) methanol:water for 10 minutes. All matrix effect experiments were made up to 500 µL.

Mixed calibration standards of the 21 amino acids were prepared by the serial dilution of the stock mixture with 50 % (v:v) methanol:water. All calibration standards were run in triplicate covering a concentration range of 0.002 – 2 ng/µL. Ten standards for the instrument precision experiment were prepared from the 21 amino acid stock mixture and run in sequence at a concentration of 40 ng/µL.

6.2.3 LC-MS instrumentation

6.2.3.1 Triple quadrupole MS

An Agilent 1100 HPLC system (Palo Alto, USA) equipped with a solvent degasser unit, a quaternary pump and a 100 well-plate autosampler was used for the chromatographic work. A Phenomenex (Torrence, USA) Gemini C18 column (250 mm x 3 mm I.D, 3 µm particle size) was used at a flow rate of 150 µL/min unless stated otherwise. Further LC conditions and parameters are detailed in Table 6.1.

Table 6.1 LC parameters for the separation of amino acids.

Time (mins)	Eluent A (%)	Eluent B (%)	Flow rate (µL/min)	LC conditions
0.00	10.0	90.0	150	Eluent A: Methanol with 0.1 % formic acid
10.00	10.0	90.0	150	
20.00	100.0	0.0	150	Eluent B: Ultrapure water with 0.1 % (v/v) formic acid
30.00	100.0	0.0	150	
31.00	10.0	90.0	200	Column: Gemini C18 column (250 mm x 3 mm I.D, 3 µm particle size)
44.00	10.0	90.0	200	
44.10	10.0	90.0	150	
45.00	10.0	90.0	150	Injection volume: 25 µL

This LC was coupled to a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Manchester, UK). The ions were generated using an ESI source operated in positive ion mode. Please refer to Table 6.2 for all ESI and MS tuning parameters. A low pH enhances the protonation of analytes in +ESI mode and is effective at ionising analytes with weak basic functional groups [251].

Nitrogen gas was used as the desolvation and nebuliser gas (BOC Gases, Australia), whereas high purity argon gas (99.997 %) was used as the collision gas for MRM experiments (BOC Gases, Australia). The parent to product ion transitions were selected based on the MS/MS spectra (Table 6.3).

Table 6.2 ESI (+) and MS tuning parameters for amino acid detection

ESI-MS tuning parameters	**Setting**
Capillary voltage (V)	3250
Cone voltage (V)	25
Hex. 1, aperture, hex. 2 (V)	0.0, 0.1, 0.2
Source temperature (°C)	135
N_2 cone gas flow (L/hr)	345
N_2 desolvation gas flow (L/hr)	47
Quad. 1 and quad. 2 resolution	1
Ion energy quad. 1 (arb) Ion energy quad. 2 (arb)	1.5 1.5
Multiplier (V)	750

arb: arbitrary units

Table 6.3 Mass Spectrometry and SRM/MRM settings for the analysis of amino acids

Amino acid	Retention time (min)	Parent ion (*m/z*)	Product ions (*m/z*)	CE (eV)	Dwell time (s)	Internal Standard
Window 1 (0-11 min)						
Lysine	5.6	147.2	84.3	15	0.080	Glutamic-d_3 acid
Ornithine	5.6	133.2	116.2 70.3	10 15	0.080	Glutamic-d_3 acid
Arginine	5.6	175.2	70.3	15	0.080	Glutamic-d_3 acid
Histidine	5.6	156.2	110.3	12	0.080	Glutamic-d_3 acid
Glycine-d_2	7.2	78.3	78.29	1	0.080	N.A.
Glycine	7.2	76.3	76.29	1	0.080	Glycine-d_2
Cysteine	7.2	122.2	76.3	15	0.080	Glutamic-d_3 acid
Alanine-d_3	7.3	93.3	47.5	10	0.080	N.A.
Alanine	7.3	90.1	44.5	10	0.080	Alanine-d_3
Serine	7.4	106.1	60.4	7	0.080	Glutamic-d_3 acid
Asparagine	7.4	133.1	74.2	15	0.080	Glutamic-d_3 acid
Glutamine	7.5	147.2	84.3	15	0.080	Glutamic-d_3 acid
Threonine	7.5	120.1	102.2	7	0.080	Glutamic-d_3 acid
Glutamic-d_3 acid	7.8	151.2	86.3	15	0.080	N.A.
Glutamic acid	7.8	148.1	102.2	11	0.080	Glutamic-d_3 acid
Aspartic acid	8.1	134.2	88.3	10	0.080	Glutamic-d_3 acid
Proline	8.2	116.1	70.2	12	0.080	Glutamic-d_3 acid
Valine	8.6	118.1	72.3	12	0.080	Glutamic-d_3 acid
Methionine	10.1	150.2	133.3 104.3	10 10	0.080	Leucine-d_3
Valine	8.6	118.1	72.3	12	0.080	Glutamic-d_3 acid
Methionine	10.1	150.2	133.3 104.3	10 10	0.080	Leucine-d_3
Isoleucine	11.1	132.2	86.3 69.4	10 17	0.080	Leucine-d_3
Leucine-d_3	11.7	135.2	89.2 30.6	10 18	0.080	N.A.
Leucine	11.9	132.2	86.3 69.4	10 17	0.080	Leucine-d_3
Tyrosine	13.2	182.2	163.3	10	0.080	Leucine-d_3
Phenyl-d_5-alanine-d_3	21.7	174.3	128.0 157.1	15 10	0.080	N.A.
Phenylalanine	22.6	166.2	102.2 131.2	15 12	0.080	Phenyl-d_5-alanine-d_3
Tryptophan	28.9	205.2	146.0 188.0	12 10	0.080	Phenyl-d_5-alanine-d_3

***NB:** CE: collision energy; N.A.: not available

6.2.3.2 Orbitrap MS

A Thermo Fisher Scientific Accela Autosampler (Massachusetts, USA), equipped with a solvent degasser unit, a quaternary pump and a 100 well-plate autosampler, was used for the chromatographic work. The chromatographic conditions were as described above, see Table 6.1.

This LC was coupled to a Thermo Fisher Scientific LTQ Orbitrap XL Linear Ion Trap Mass Spectrometer (Massachusetts, USA). The ions were generated using an ESI source operated in positive ion mode. Please refer to Table 6.4 for all ESI and MS tuning parameters.

Nitrogen gas was used as the desolvation and nebuliser gas (BOC Gases, Australia). The retention times, parent ion *m/z* ratios and the corresponding internal standards of each amino acid are presented in Table 6.5.

Table 6.4 ESI (+) and MS tuning parameters for amino acid detection

ESI-MS tuning parameters	**Setting**
Spray voltage (kV)	3.5
Capillary voltage (V)	25
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	-110
Scan range (*m/z*)	50-250
IT full MS AGC target	3E4
IT MS^n AGC target	1E4
FT full MS AGC target	2E5
FT MS^n AGC target	1E5
Ion trap and FT micro scans	3
IT full MS Max ion time (ms)	10
FT MS^n Max ion time (ms)	100
MS^2 isolation window (*m/z*)	1

Table 6.5 Parent ions (+ESI) mass tuning parameters of amino acids.

Amino acid	Retention time (min)	Parent ion (*m/z*)	Internal Standard
Lysine	5.3	147.1128	Glutamic-d_3 acid
Arginine	5.4	175.1190	Glutamic-d_3 acid
Histidine	5.4	156.0768	Glutamic-d_3 acid
Ornithine	5.4	133.0972	Glutamic-d_3 acid
Glycine	6.9	76.0391	Glycine-d_2
Glycine-d_2	6.9	78.0519	N.A.
Alanine	7.0	90.0550	Alanine-d_3
Alanine-d_3	7.0	93.0738	N.A.
Serine	7.0	106.0499	Glutamic-d_3 acid
Asparagine	7.1	133.0608	Glutamic-d_3 acid
Glutamine	7.2	147.0764	Glutamic-d_3 acid
Threonine	7.2	120.0655	Glutamic-d_3 acid
Glutamic acid	7.5	148.0604	Glutamic-d_3 acid
Glutamic-d_3 acid	7.5	151.0793	N.A.
Aspartic acid	7.8	134.0448	Glutamic-d_3 acid
Proline	7.8	116.0706	Glutamic-d_3 acid
Cysteine	8.1	122.0270	Glutamic-d_3 acid
Valine	8.3	118.0863	Glutamic-d_3 acid
Methionine	9.7	150.0583	Leucine-d_3
Isoleucine	11.2	132.1019	Leucine-d_3
Leucine-d_3	11.8	135.1207	N.A.
Leucine	12	132.1019	Leucine-d_3
Tyrosine	12.8	182.0812	Leucine-d_3
Phenyl-d_5-alanine-d_3	21.5	174.1365	N.A.
Phenylalanine	21.9	166.0863	Phenyl-d_5-alanine-d_3
Tryptophan	23.8	205.0972	Phenyl-d_5-alanine-d_3

***NB:** N.A.: not available

6.2.4 Statistical analyses

The Mann-Whitney U test and Wilcoxon signed rank test analyses were performed with IBM's SPSS Version 2.0. Principal component analysis (PCA) was performed with the Unscrambler® X 10.3 Software (CAMO Software AS, Oslo, Norway).

6.3 Results and discussion

The method developed by Swann *et al.* was used as a starting point for this work [214]. In that application the LC-MS method was used for the determination of amino acid content in decaying pig tissue. It has since been updated and revised for a wider range of amino acids in a water matrix by How *et al.*, where the results point to a robust and reproducible method [252]. In contrast to the method used by de Puit *et al.*, this method does not rely on derivatisation prior to analysis. Derivatisation results in improved detection limits and potentially better fit of the calibration curve, as both the selectivity and sensitivity can suffer with certain underivatised analytes. However, the simplicity of sample preparation and the success of the method in other applications, make the use of underivatised samples appealing. In addition to the reduced sample preparation, the time taken for complete elution is also much shorter (at 45 minutes) compared to the method proposed by de Puit *et al.* (67 minutes), reducing solvent usage and waste, and overall analysis time. Fingermark samples from 3 donors were analysed without modification to the method described by How *et al.* [252]. Separation of the target amino acids was achieved and the most abundant amino acids (as published previously) were detected (Figure 6.1) [34]. The existing method was further developed to include l-ornithine and adapted to be more amenable for the analysis of amino acids extracted from fingermarks deposited on a porous substrate.

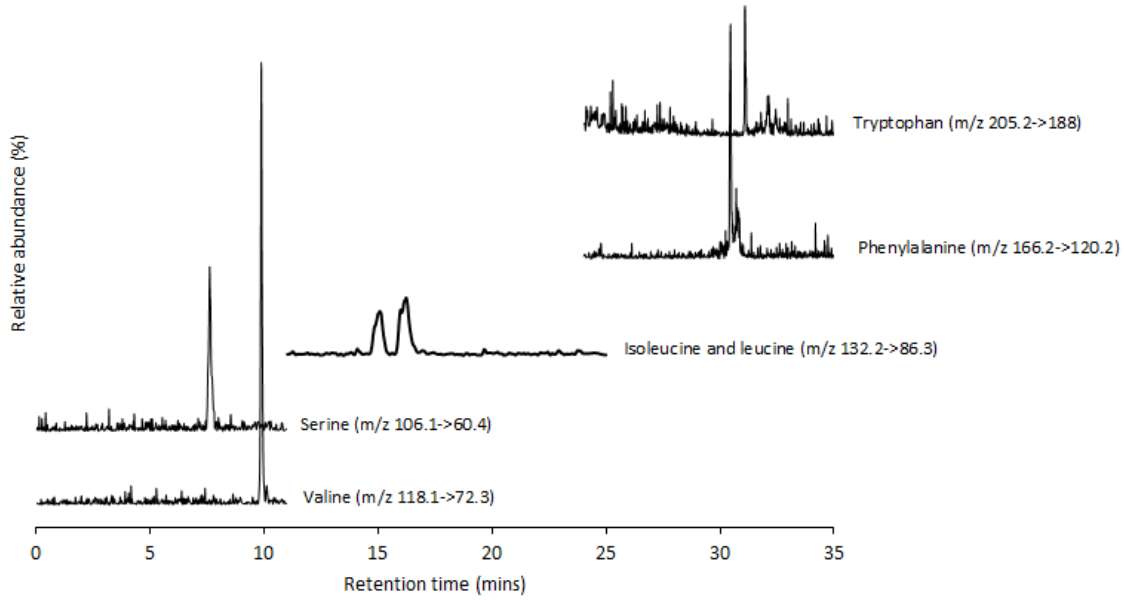


Figure 6.1 Extracted ion LC-MS chromatograms of selected amino acids detected in window 1-3 of a latent fingermark residue. Amino acids were analysed using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and a triple quadrupole mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode.

6.3.2 Method development

The first alteration to the existing method was to include l-ornithine to the target list of amino acids. Direct infusion experiments were used to optimise general MS and MS/MS tuning parameters. A single compound standard solution (10 ng/µL) prepared in 50:50 (v:v) mixture of eluent A (methanol with 0.1 % formic acid) and ultrapure water was used. In order to ascertain the product ions arising from the fragmentation of l-ornithine, the parent ion (*m/z* 133.2) was subjected to increasing collision energy and the spectrum was recorded. 10 eV collision energy caused an abundance of 116.2 *m/z* product ions, while 15 eV resulted in product ions of *m/z* 70.3. Although asparagine has a very similar *m/z* (133.1→product) transition, the two amino acids could be distinguished based upon their chromatographic retention times and fragmentation ions (Figure 6.2, Table 6.3).

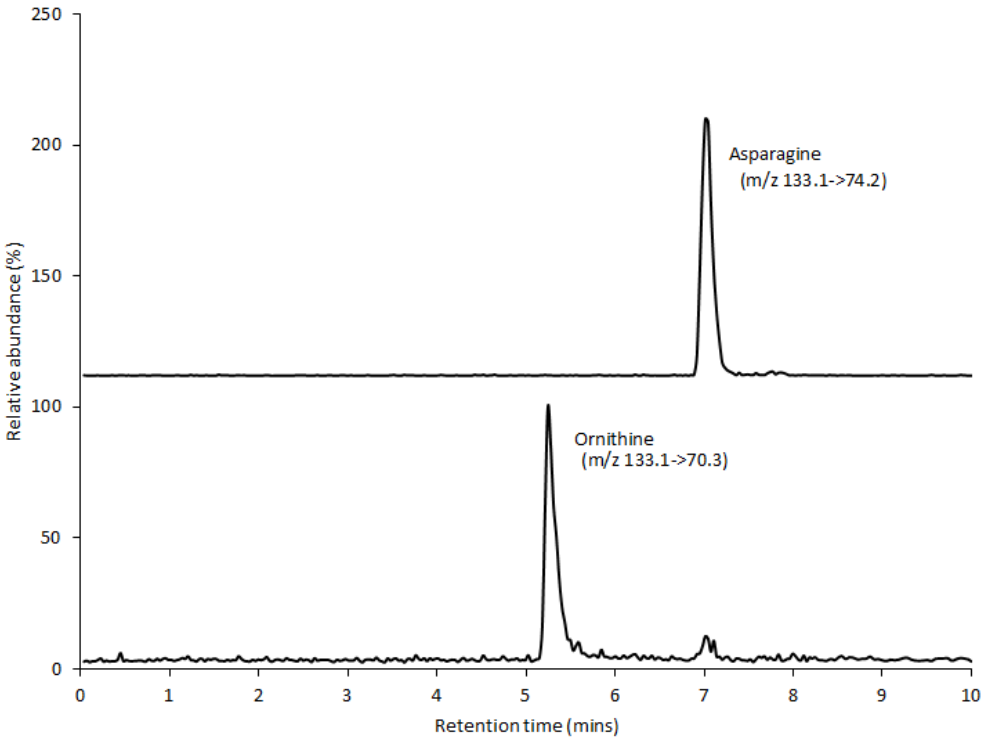


Figure 6.2 Extracted ion LC-MS chromatograms of ornithine (5.25 min) and asparagine (7.01 min) separation from an amino acid mixture despite similar *m/z* ratios (133.2 and 133.1, respectively) due to the retention times and product ions. Amino acids were analysed using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and a triple quadrupole mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode.

In this study, complete chromatographic separation was not achieved for all amino acids. However, due to the very selective nature of MS detectors, sufficient separation was only required for those amino acids with very similar molecular or product ions. A further benefit is that the mass analyser scans through all analytes for 0.5 seconds, and increasing the number of data points per signal gives a smoother peak shape. To enhance this effect, the analytes were therefore separated into 2 windows. Each window has a smaller number of amino acids to be analysed, resulting in a limited number of parent to product transitions to recorded, where each analyte is scanned more frequently. Window 1 consisted of amino acids eluting within the first 10 minutes, and analytes eluting between 10 - 30 minutes were scanned for in window 2 (Table 6.3). A sample chromatogram of the combined windows resulting from the analysis of the 21 amino acid stock solution is shown in Figure 6.3.

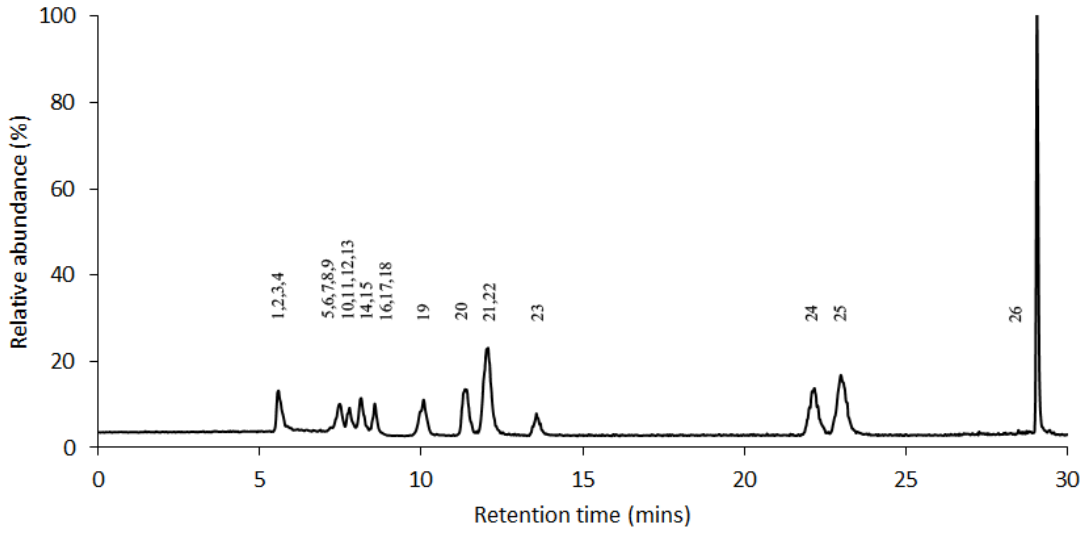


Figure 6.3 Total ion current LC-MS chromatogram of amino acids detected in window 1 and 2 of a standard amino acid and IS mixture. Amino acids were analysed using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and a triple quadrupole mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode. Peak assignment: 1) Lysine, 2) ornithine, 3) arginine, 4) histidine, 5) glycine-d_2 (IS), 6) glycine, 7) cysteine, 8) alanine-d_3 (IS), 9) alanine, 10) serine, 11) asparagine, 12) glutamine, 13) threonine, 14) glutamic-d_3 acid (IS), 15) glutamic acid, 16) aspartic acid, 17) proline, 18) valine, 19) methionine, 20) isoleucine, 21) leucine-d_3 (IS), 22) leucine, 23) tyrosine, 24) phenyl-d_5-alanine-d_3 (IS), 25) phenylalanine, 26) tryptophan.

***NB:** IS: internal standard

6.3.2.1 Sample deposition and extraction

The sample deposition and extraction methods were investigated as described in Chapter 5, where the fingermark collection media was again filter paper circles to offer consistency between the HPLC-UV-DAD and LC-MS analyses. Very high extraction efficiency was achieved using 50 % (v:v) methanol:water for 1 hour without agitation (Table 6.6), in addition to offering much more consistent results (relative standard deviation of 4.90 %) than the other extraction methods tested in this study (Appendix C). These results mirror the findings for the HPLC-UV-DAD analysis of amino acids in Chapter 5, where agreement between the two extraction methods was vital so that each sample could be analysed on both instruments.

Table 6.6 Relative extraction efficiencies of the various extraction methods (%).

	Extraction efficiency (%)							
	30 % (v:v) methanol:water				**50 % (v:v) methanol:water**			
Amino acid	**Agitation 5 mins**	**Agitation 10 mins**	**No agitation 30 mins**	**No agitation 60 mins**	**Agitation 5 mins**	**Agitation 10 mins**	**No agitation 30 mins**	**No agitation 60 mins**
Alanine	98.1	93.9	99.1	94.4	100.0	86.5	91.8	89.3
Alanine-d_3	69.7	82.6	63.7	52.8	69.7	100.0	75.1	80.1
Arginine	80.5	76.9	90.1	86.3	62.4	71.4	100.0	90.9
Asparagine	96.8	94.4	100.0	92.5	95.8	95.4	91.8	83.5
Aspartic acid	94.4	92.0	100.0	98.4	89.9	89.7	87.5	80.9
Cysteine	88.5	100.0	52.9	88.7	88.4	89.8	77.0	86.2
Glutamic acid	92.7	92.6	100.0	99.0	99.2	93.2	94.6	94.2
Glutamic-d_3 acid	73.8	88.0	69.6	64.0	70.4	100.0	76.1	70.8
Glutamine	98.1	93.9	100.0	92.7	90.9	90.7	88.4	82.1
Glycine	87.5	93.0	90.7	100.0	91.0	90.9	97.1	93.9
Glycine-d_2	72.9	85.5	68.6	52.2	75.4	100.0	77.9	86.5
Histidine	75.6	77.7	87.5	90.0	78.6	77.2	100.0	97.8
Isoleucine	72.6	73.2	97.0	100.0	71.0	70.2	77.0	68.1
Leucine	72.6	73.2	97.0	100.0	71.0	70.2	77.0	68.1
Leucine-d_3	67.3	81.0	66.4	56.1	73.0	100.0	76.6	76.1
Lysine	97.8	93.6	100.0	92.5	90.6	90.4	88.0	81.7
Methionine	91.2	83.0	89.8	100.0	77.5	0.0	86.7	77.9
Ornithine	85.2	79.8	94.2	100.0	46.2	62.7	88.3	82.2
Phenylalanine	70.9	100.0	69.8	59.3	71.3	74.9	87.1	99.0
Phenyl-d_5-alanine-d_3	68.9	83.2	71.8	66.9	71.4	100.0	82.3	75.4
Proline	86.6	85.3	88.3	100.0	79.9	78.9	86.6	78.9
Serine	87.7	104.2	88.5	83.6	88.6	120.4	100.0	91.2
Threonine	93.0	94.6	95.2	90.7	98.0	100.0	97.8	92.2
Tryptophan	72.0	82.7	74.4	69.4	77.0	99.8	100.0	87.7
Tyrosine	92.7	91.8	99.2	100.0	86.8	86.3	95.3	91.4
Valine	71.5	85.3	73.7	60.9	69.7	100.0	67.4	60.4
Average	*83.0*	*87.7*	*85.7*	*84.2*	*80.2*	*86.1*	*87.2*	*83.3*

6.3.2.2 Matrix effect

In ESI-MS methods, matrix effects can occur which may cause ion enhancement, but usually ion suppression, in samples due to the co-eluting matrix [214, 253]. This can manifest itself in decreased analytical accuracy and linearity, as well as reduced reproducibility [253]. To test for any matrix effect, an amino acid standard solution was compared to amino acid mixture extracts from filter paper using either a 50 % (v:v) methanol:water soak for 1 hour, or sonication with 50 % (v:v) methanol:water for 10 minutes. No observable matrix effect was evident, and the results from the amino acid to IS ratios of the standard solution are very similar to those of the two different filter paper extraction methods (Figure 6.4).

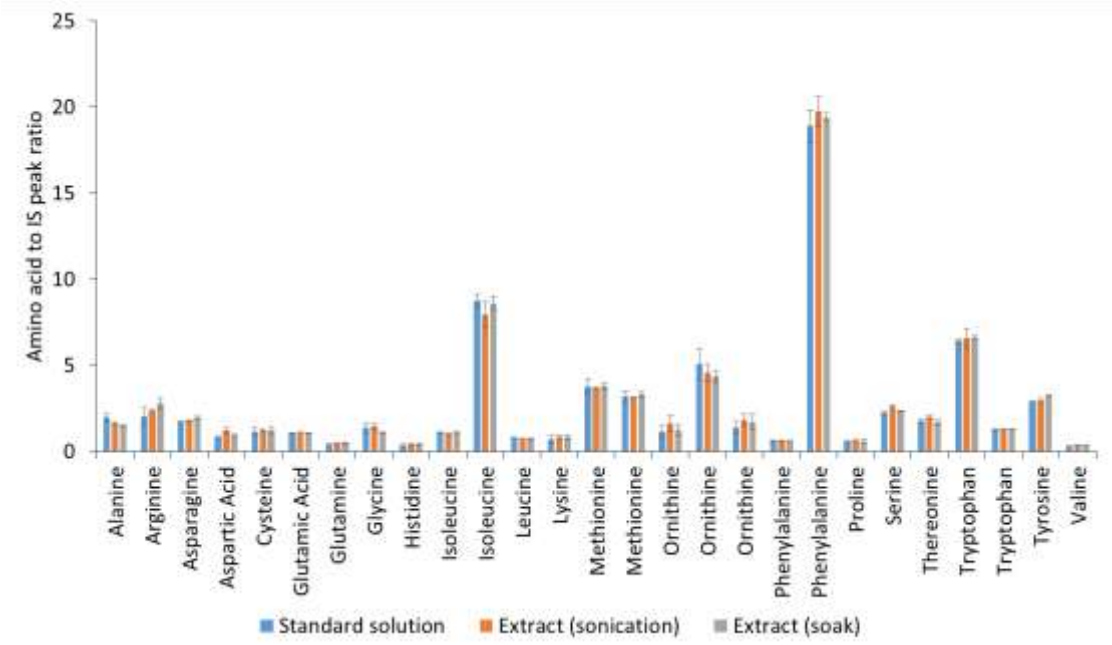


Figure 6.4 Bar chart displaying the amino acid to corresponding IS peak area ratio for the molecular and fragment ions.

6.3.2.3 Sensitivity

Changes to the sample volume (300 µL) would offer limited increases in the detector response (as opposed to HPLC-UV-DAD where the samples are reconstituted), as a minimum sample volume of ~200 µL is required for the auto-injector. At this stage, the introduction of a drying and reconstitution step, and their associated sample preparation risks, was not deemed appropriate for an increase in concentration of 33 %. The LC method was therefore adjusted by simply increasing the injection

volume from 2.5 to 10 µL and then again to 25 µL, where the tenfold increase in analyte amount did not overload the column nor affect the detector response in a negative manner. As can be seen with serine in Figure 6.5, the largest injection volume was followed by the largest detector response.

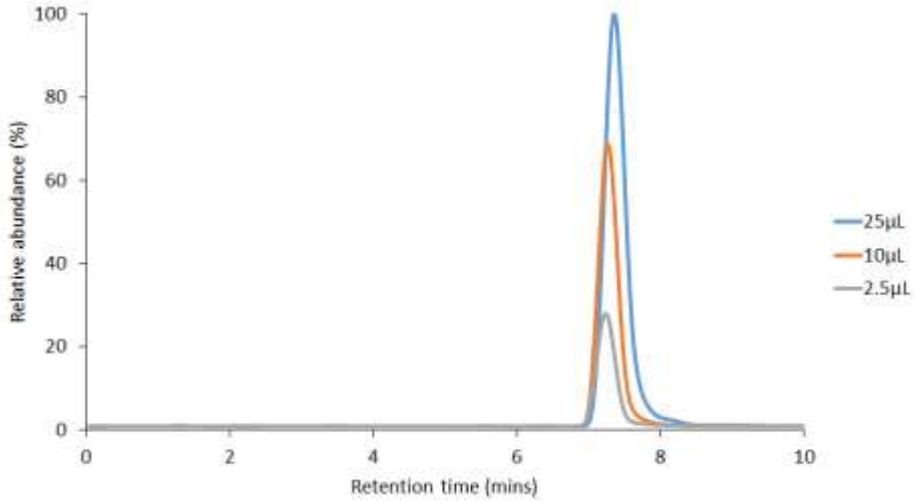


Figure 6.5 Extracted ion LC-MS chromatograms highlighting the differences in injection volume and the subsequent detector response of serine (*m/z* 106.1→60.4). Amino acids were analysed using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and a triple quadrupole mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode.

6.3.2.4 Instrument issues

Upon completion of the method development, and some of the validation work outlined below, the LC-MS instrument outfitted with the triple quadrupole mass analyser developed electrical issues following a power surge. Exchanging the affected circuit board did not result in a working instrument, and much more expensive maintenance would possibly be required. In addition to the costs involved, it was not possible to wait for a resolution to this instrument's issues due to the very strict time constraints attributed to this project. A LC-MS instrument coupled to an Orbitrap mass analyser was therefore used for all subsequent sample analyses.

6.3.2.5 Orbitrap MS

For the detection of amino acids using the Orbitrap mass analyser, all MS settings had to be revised and developed. The sample preparation procedure and LC conditions could be retained from the triple quadrupole instrument as the same separation column was used. The only change that was made to the LC conditions

was a reduction in injection volume from 25 to 10 µL. Using the standard 21 amino acid and internal standard solution in direct injection mode, the CE voltages were tuned for each amino acid to offer the best detectability (Table 6.5).

Detection of amino acids using the Orbitrap mass analyser was achieved in target data-dependant MS scan mode, where the MS is set to acquire the MS scan spectra at 30000 resolving power followed by the MS2 spectra of target amino acids precursors at 7500 resolving power. The target data-dependant MS scan mode gives valuable information on the accurate mass (i.e. elemental formula) of the parent ion and fragments for the analytes of interest. Multiple detection windows are often not necessary and the chromatographic conditions offer separation of amino acids which have identical *m/z* ratios, such as leucine and isoleucine (Figure 6.6).

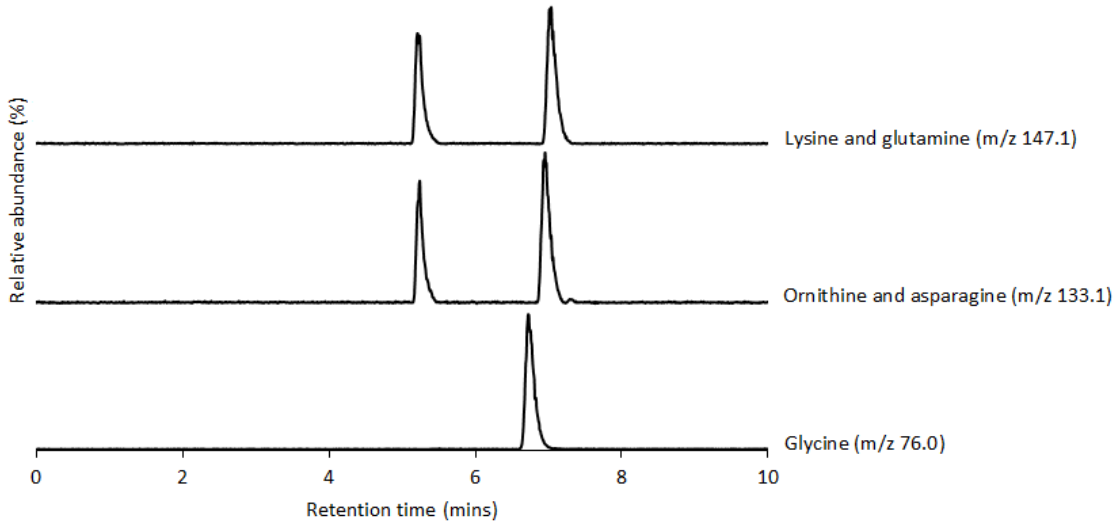


Figure 6.6 Extracted ion LC-MS chromatograms of selected amino acids using the Orbitrap mass analyser. Amino acids were separated using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size).

6.3.3 Method validation

Method validation was performed for both the triple quadrupole and Orbitrap mass analyser instruments. The signal to noise ratio (S/N) was used to estimate the LOD (S/N > 3) and LLOQ (S/N > 10) (Table 6.7). The linear calibration range for the Orbitrap was established to lie within 0.002 and 0.8 ng/µL, where the average correlation coefficient was found to be 0.993 (Table 6.7). Asparagine was quantified at concentrations above 0.004 ng/µL, histidine and lysine at concentrations above 0.008 ng/µL, and arginine and ornithine at concentrations above 0.04 ng/µL.

Good precision was displayed with 10 replicates at stock solution concentration with analysis of both the standard solution and the extraction of the standard solution from filter paper. The average precision of standard solutions spotted directly into the vial was found to be 9.5 % for the triple quadrupole and 10.6 % for the Orbitrap (as a function of % relative standard deviation (RSD)), well below the acceptable RSD of 15 % for precision experiments [220]. As expected, the precision of extracted samples was lower, where values of 12.6 % and 10.7 %, respectively, were obtained (Table 6.7). Cysteine is omitted from these results on account of its very poor performance with the proposed LC-MS methods. The average extraction efficiency was shown to be 82 %, where the type of amino acid again determined its exact recovery (Table 6.7). These percentages are comparable to those achieved by the HPLC-UV-DAD method described in Chapter 5. Fresh and one year old amino acid standard solutions (stored at 4 °C) were compared and found to be very similar, where only cysteine showed signs of degradation, possibly due to oxidation.

Table 6.7 Limit of detection and lower limit of quantification for the triple quadrupole and Orbitrap mass analyser instruments.

Amino acid	Correlation coefficient (0.002-0.8 ng/µL)	Limit of detection (pg/µL)		Lower limit of quantification (pg/µL)		Precision (% RSD)		Extraction efficiency (%)
	Orbitrap	*qQq*	*Orbitrap*	*qQq*	*Orbitrap*	*qQq*	*Orbitrap*	*qQq*
Alanine	0.999	15.8	166.7	52.8	555.6	15.9	10.8	111.5
Alanine-d_3	-	35.3	219.8	117.6	732.6	7.8	8.1	93.7
Arginine	0.979	9.6	320.9	31.9	1069.5	5.9	1.6	79.5
Asparagine	0.999	3.4	729.9	11.3	2433.1	10.2	2.8	65.4
Aspartic acid	0.998	24.5	170.0	81.8	566.6	14.5	19.2	109.0
Cysteine	N/A	195.8	0.0	652.7	0.0	64.0	N/A	66.6
Glutamic acid	0.998	1.8	150.0	6.0	500.0	4.4	4.5	98.6
Glutamic-d_3 acid	-	1.4	196.7	4.6	655.7	21.0	20.2	100.8
Glutamine	1.000	4.3	209.8	14.2	699.3	7.9	13.9	79.6
Glycine	0.999	46.6	387.1	155.2	1290.3	35.3	0.7	105.1
Glycine-d_2	-	68.9	203.4	229.7	678.0	21.9	7.1	97.6
Histidine	0.997	2.5	521.7	8.4	1739.1	6.0	1.3	80.6
Isoleucine	0.996	2.7	566.0	8.8	1886.8	3.0	8.3	103.3
Leucine	0.999	1.5	123.2	4.9	410.7	3.0	12.0	103.3
Leucine-d_3	-	1.1	272.7	3.7	909.1	11.5	11.8	84.9
Lysine	0.962	2.7	134.5	8.9	448.4	5.6	20.1	79.1
Methionine	0.982	1.6	714.3	5.2	2381.0	4.9	12.9	107.5
Ornithine	0.951	9.4	38.7	31.4	129.1	10.3	19.1	102.8
Phenylalanine	1.000	2.8	96.5	9.4	321.5	5.69	16.7	111.8
Phenyl-d_5-alanine-d_3	-	1.1	41.7	3.7	138.9	10.1	0.8	95.0
Proline	0.999	2.7	192.3	8.9	641.0	14.9	12.4	105.7
Serine	0.999	10.9	159.6	36.2	531.9	9.4	12.4	103.8
Threonine	1.000	13.0	109.5	43.4	365.0	8.0	9.2	70.6
Tryptophan	1.000	0.2	131.6	0.6	438.6	7.0	18.3	116.6
Tyrosine	0.991	4.7	628.3	15.7	2094.2	3.6	17.3	102.3
Valine	1.000	2.0	98.4	6.6	327.9	16.4	5.0	98.0
Average	*0.993*	17.9	253.2	59.8	844.0	*12.6*	*10.7*	*95.1*

***NB:** Asparagine was quantified at concentrations above 0.004 ng/µL, histidine and lysine at concentrations above 0.008 ng/µL, and arginine and ornithine at concentrations above 0.04 ng/µL.

6.3.4 Fingermark sample analysis

Fingermark samples from the same 50 donors (Table 6.8) used in Chapter 5 were analysed using the LC-MS Orbitrap mass analyser instrument.

Table 6.8 Donor information with regards to the number of donors for each variable (n=50).

Variable	Grouping	Number of Donors
Biological sex	Male	*29*
	Female	*21*
Age	Over 25	*23 (M:16, F:7)*
	Under 25	*27 (M:13, F:14)*
Food Consumption	Yes	*16*
	No	*34*
Washing of hands	Yes	*10*
	No	*40*
Cosmetics	Yes	*16*
	No	*34*

Serine was again found to be the predominant analyte (Figure 6.7). This was followed by arginine, ornithine, glycine, lysine and alanine (in that order). The average concentration of each amino acid per fingermark was found to be between 1.5 ng (tryptophan) and 65.4 ng (serine), Table 6.9. The sum of the average amino acid concentrations was shown to be 363 ng per fingermark, but ranged from 66 to 1371 ng per fingermark depending on the donor. While these results are within the same order of magnitude to those presented in Chapter 5, the overall concentrations are much lower (by a factor of 1.85) with LC-MS detection. Interestingly, the LC-MS results are in much closer agreement with those by Croxton *et al.* (20.7 to 345.1 ng) and Hansen *et al.*'s estimation (250 ng) than the HPLC-UV-DAD findings. There is also a change to the order of the most abundant amino acids, where serine is followed by arginine, which was only the 8th most abundant for HPLC-UV-DAD analysis. Due to the difficulties in obtaining reliable identification of cysteine with LC-MS detection, this amino acid was not quantified and therefore omitted from further discussion. The difference between the instruments speaks of the difficult nature of quantifying these analytes at very low concentrations after extraction from a complex matrix.

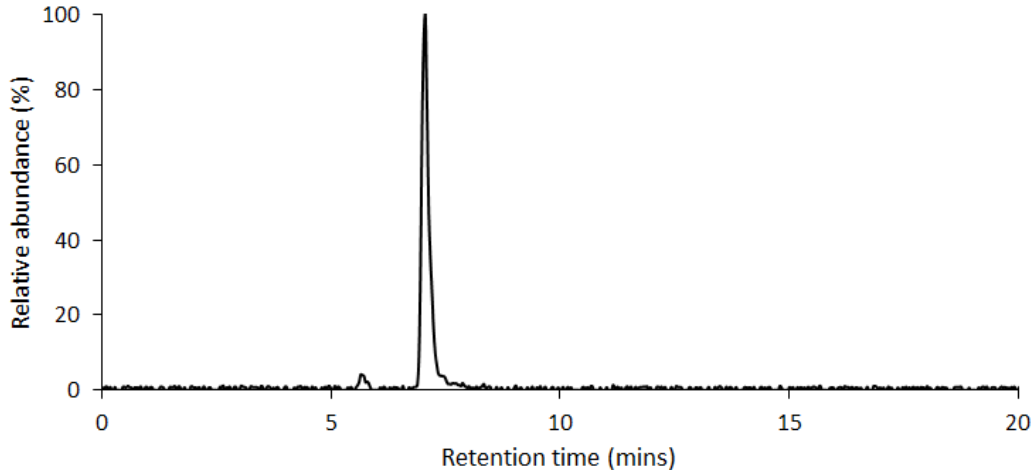


Figure 6.7 Extracted ion LC-MS chromatogram of serine (*m/z* range 105.5-106.5) in a latent fingermark sample. Serine was separated using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and an Orbitrap mass spectrometer operated in MS Scan mode.

6.3.4.1 Donor traits

Inter-donor variation was investigated by assessing possible differences in the results due to donor traits (Table 6.9). Figure 6.8 shows that the relative amino acid abundance is very similar in both sexes. The absolute concentrations are only marginally different for serine and very similar for the other 19 amino acids (Figure 6.9). HPLC-UV-DAD analysis by contrast gave very different absolute quantities for males than females, but very similar relative profiles. This difference may be due to the same instrument limitations outlined above.

Table 6.9 Abundance of amino acids, both absolute values and relative to the sum of total amino acid concentration, of donor traits.

Amino acid	Absolute concentration (ng/fingermark)					Relative concentration (%)				
	All donors	Female donors	Male donors	Under 25	25 and over	All donors	Female donors	Male donors	Under 25	25 and over
Alanine	21.0	18.0	23.4	23.4	18.3	5.8	5.7	5.9	6.3	5.2
Arginine	50.4	48.0	52.2	53.4	47.1	13.9	15.2	13.2	14.3	13.5
Asparagine	7.5	6.3	8.1	7.5	7.2	2.1	2.0	2.0	2.0	2.1
Aspartic acid	17.7	14.7	20.1	16.2	19.8	4.9	4.7	5.1	4.3	5.7
Glutamic acid	9.6	9.0	10.2	10.2	9.0	2.6	2.9	2.6	2.7	2.6
Glutamine	5.1	4.8	5.1	5.1	4.8	1.4	1.5	1.3	1.4	1.4
Glycine	37.5	32.1	41.1	42.6	31.5	10.3	10.2	10.4	11.4	9.0
Histidine	13.2	10.8	15.0	13.8	12.6	3.6	3.4	3.8	3.7	3.6
Isoleucine	6.0	5.1	6.6	6.0	6.0	1.7	1.6	1.7	1.6	1.7
Leucine	4.8	3.6	5.7	4.5	5.1	1.3	1.1	1.4	1.2	1.5
Lysine	35.1	33.0	36.6	36.6	33.3	9.7	10.5	9.2	9.8	9.5
Methionine	4.8	3.6	5.4	4.8	4.5	1.3	1.1	1.4	1.3	1.3
Ornithine	37.5	33.3	40.2	35.1	40.2	10.3	10.6	10.1	9.4	11.5
Phenylalanine	2.4	1.8	2.7	1.8	2.7	0.7	0.6	0.7	0.5	0.8
Proline	9.9	8.1	11.4	11.4	8.4	2.7	2.6	2.9	3.0	2.4
Serine	65.4	54.9	73.2	66.3	64.5	18.0	17.4	18.5	17.7	18.4
Threonine	19.8	17.1	21.9	21.9	17.4	5.5	5.4	5.5	5.9	5.0
Tryptophan	1.5	1.5	1.5	1.2	2.1	0.4	0.5	0.4	0.3	0.6
Tyrosine	5.7	3.6	7.5	4.8	7.2	1.6	1.1	1.9	1.3	2.1
Valine	7.8	6.3	8.7	7.2	8.1	2.2	2.0	2.2	1.9	2.3

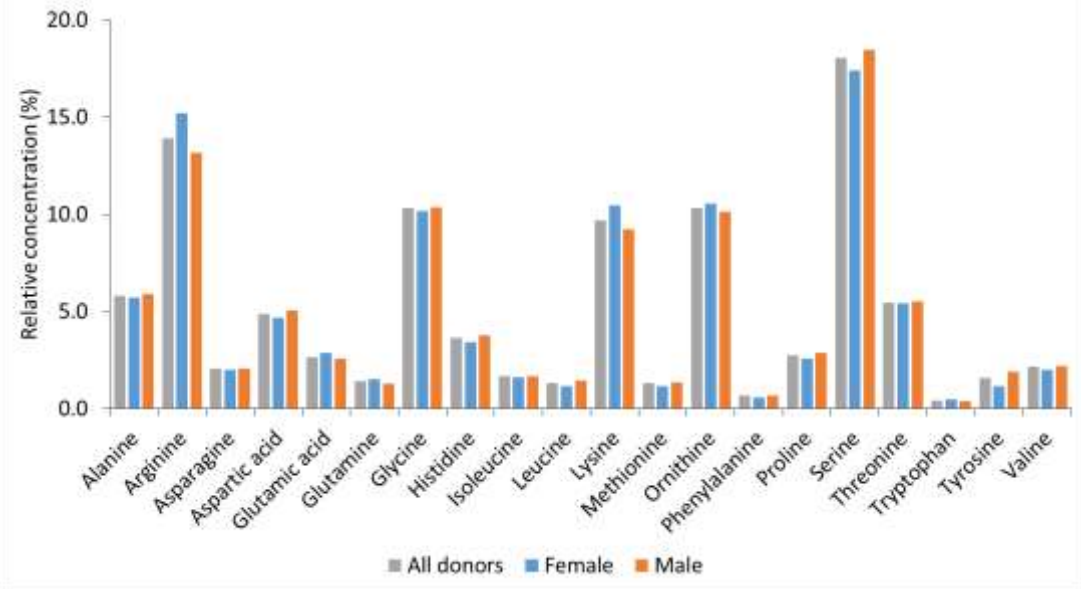


Figure 6.8 Relative, to the sum of total amino acid concentration, abundance of amino acids in all donors, males, and females.

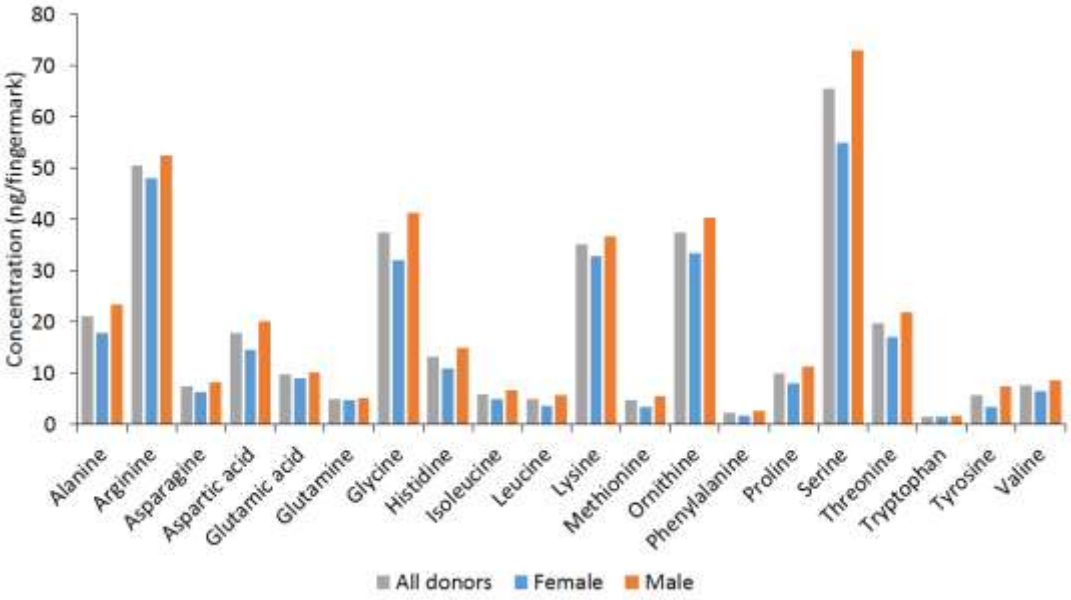


Figure 6.9 Absolute abundance of amino acids in all donors, males, and females.

The greatest differences between donors over and under the age of 25 were shown in the glycine, ornithine, arginine and aspartic acid content with both the relative and absolute concentrations (Figure 6.10 and 6.11 respectively). While there was a marked difference in the absolute concentration for HPLC-UV-DAD results, where amino acids were much more abundant in younger donors, no such trend could be established with LC-MS analysis.

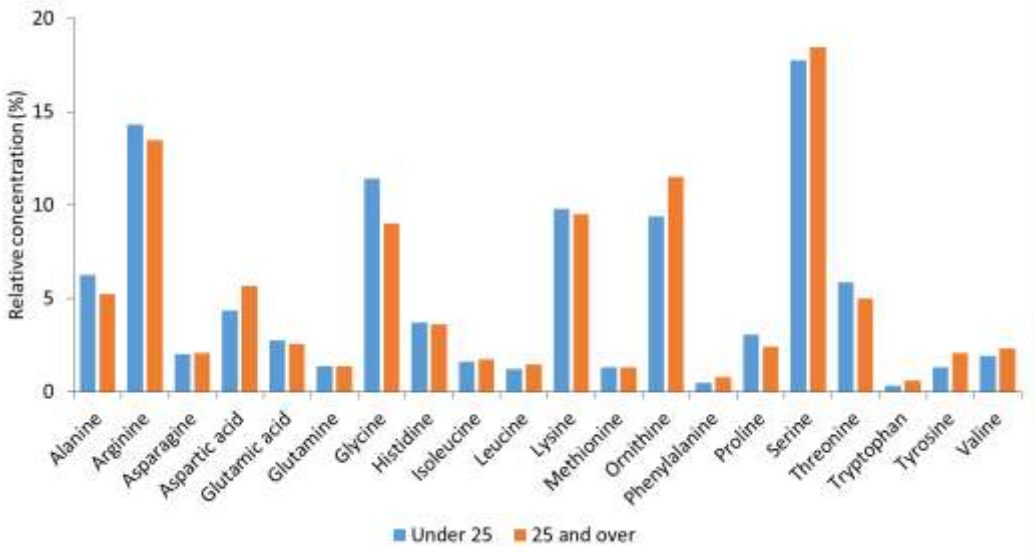


Figure 6.10 Relative, to the sum of total amino acid concentration, abundance in donors under and over the age of 25 years.

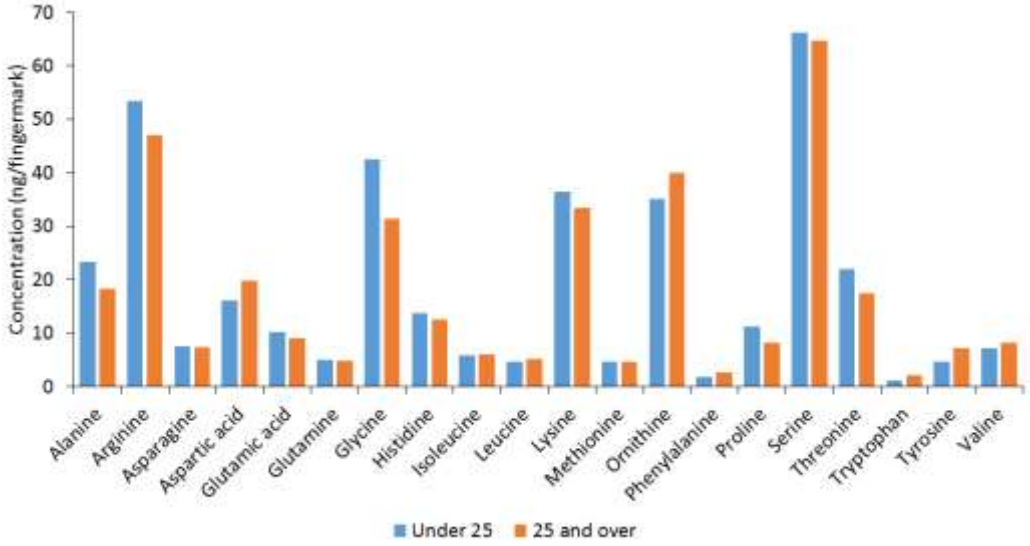


Figure 6.11 Absolute abundance of amino acids in donors under and over the age of 25 years.

6.3.4.1 Donor habits

In addition to the donor traits discussed above, the effect of donor habits on the amino acid content were examined (Table 6.10). The major differences between donors that had washed their hands to those that had not within the hour prior to fingermark deposition are in the relative amounts of serine, arginine, lysine, ornithine, alanine and aspartic acid, i.e. in the profile of the most abundant amino acids (Figure 6.12). The absolute concentration profile showed even larger differences (Figure 6.13). Donors who had washed their hands had a much lower concentration of all amino acids, as to be expected from the water soluble nature of these analytes. These results mirror the conclusion drawn in Chapter 4, where 1,2-indanedione/zinc chloride gave a weaker response to deposits from donors who had washed their hands. In addition, these findings are also in agreement with Chapter 5, where HPLC-UV-DAD analysis observed similar trends.

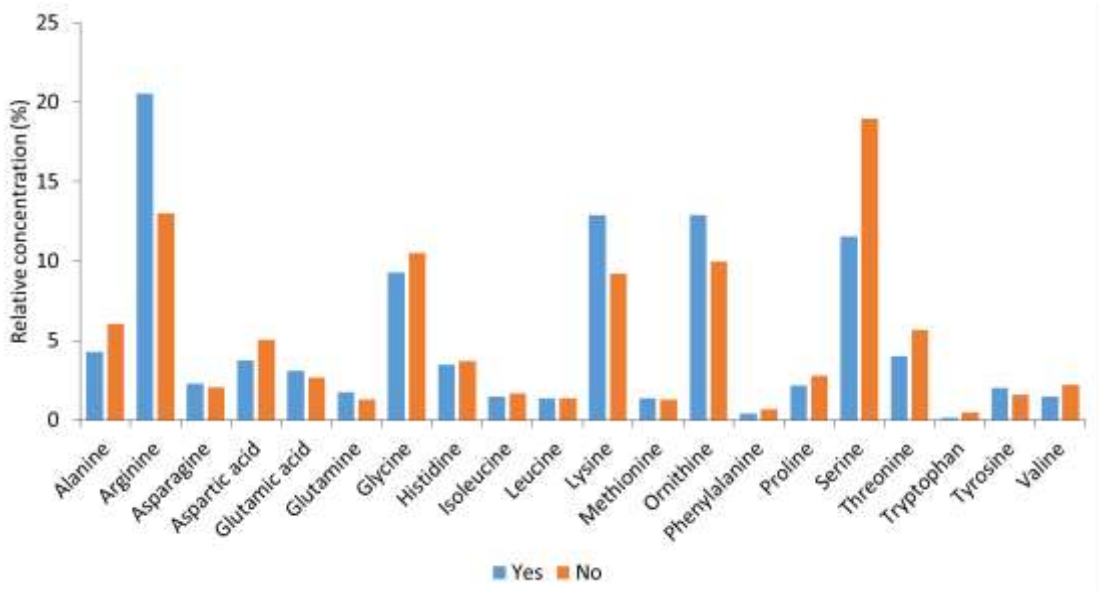


Figure 6.12 Relative, to the sum of total amino acid concentration, abundance in donors who had and had not washed their hands within one hour of fingermark deposition.

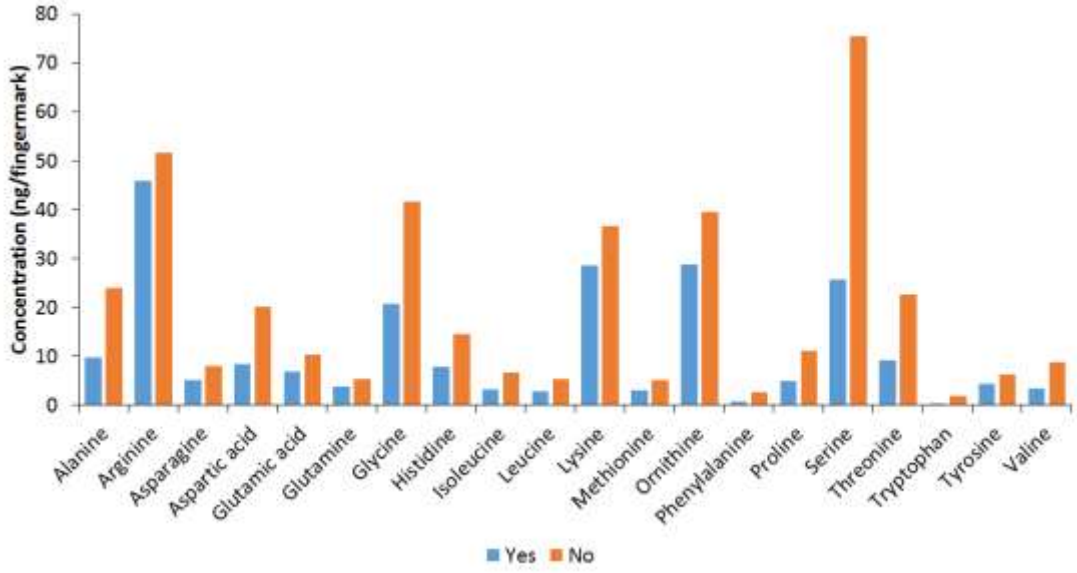


Figure 6.13 Absolute abundance of amino acids in donors who had and had not washed their hands within one hour of fingermark deposition.

Table 6.10 Abundance of amino acids, both absolute values and relative to the sum of total amino acid concentration, of donor habits.

Amino acid	Absolute concentration (ng/fingermark)						Relative concentration (%)					
	Washed hands	Unwashed hands	Food consumed	No food consumed	Cosmetics used (<12 hrs)	No cosmetics used (<12 hrs)	Washed hands	Unwashed hands	Food consumed	No food consumed	Cosmetics used (<12 hrs)	No cosmetics used (<12 hrs)
Alanine	9.6	24.0	19.8	21.6	18.6	22.2	4.3	6.0	5.9	5.8	5.4	6.0
Arginine	45.9	51.6	48.6	51.3	47.4	51.9	20.5	13.0	14.4	13.7	13.9	13.9
Asparagine	5.1	8.1	7.5	7.5	6.9	7.8	2.3	2.0	2.2	2.0	2.0	2.1
Aspartic acid	8.4	20.1	16.8	18.3	17.4	18.0	3.8	5.1	5.0	4.9	5.1	4.8
Glutamic acid	6.9	10.5	9.6	9.9	9.3	9.9	3.1	2.6	2.8	2.6	2.7	2.7
Glutamine	3.9	5.1	5.1	4.8	5.1	5.1	1.7	1.3	1.5	1.3	1.5	1.4
Glycine	20.7	41.7	36.0	38.1	31.8	40.2	9.2	10.5	10.6	10.2	9.3	10.8
Histidine	7.8	14.7	12.6	13.5	12.6	13.5	3.5	3.7	3.7	3.6	3.7	3.6
Isoleucine	3.3	6.6	5.4	6.3	6.0	6.0	1.5	1.7	1.6	1.7	1.8	1.6
Leucine	3.0	5.4	4.2	5.1	4.8	4.8	1.3	1.4	1.2	1.4	1.4	1.3
Lysine	28.8	36.6	33.0	36.0	33.6	35.7	12.9	9.2	9.8	9.6	9.8	9.6
Methionine	3.0	5.1	4.2	4.8	5.1	4.5	1.3	1.3	1.2	1.3	1.5	1.2
Ornithine	28.8	39.6	34.2	39.0	36.9	37.5	12.9	10.0	10.1	10.4	10.8	10.1
Phenylalanine	0.9	2.7	1.8	2.4	2.7	2.1	0.4	0.7	0.5	0.6	0.8	0.6
Proline	4.8	11.1	9.3	10.2	8.1	10.8	2.1	2.8	2.8	2.7	2.4	2.9
Serine	25.8	75.3	59.1	68.4	61.5	67.2	11.5	18.9	17.5	18.3	18.0	18.0
Threonine	9.0	22.5	18.0	20.7	17.4	21.0	4.0	5.7	5.3	5.5	5.1	5.6
Tryptophan	0.3	1.8	0.9	1.8	2.1	1.5	0.1	0.5	0.3	0.5	0.6	0.4
Tyrosine	4.5	6.3	5.4	6.0	6.6	5.4	2.0	1.6	1.6	1.6	1.9	1.4
Valine	3.3	8.7	6.6	8.1	7.5	7.8	1.5	2.2	2.0	2.2	2.2	2.1

Serine, lysine, histidine and arginine were the four amino acids that showed the most significant difference in their relative concentrations between donors who had and had not recently consumed food (Figure 6.14). Except for asparagine and glutamine, all amino acids were found to be more abundant in donors who had consumed food compared to those that had not (Figure 6.15). The largest difference was observed in the concentrations of serine and ornithine.

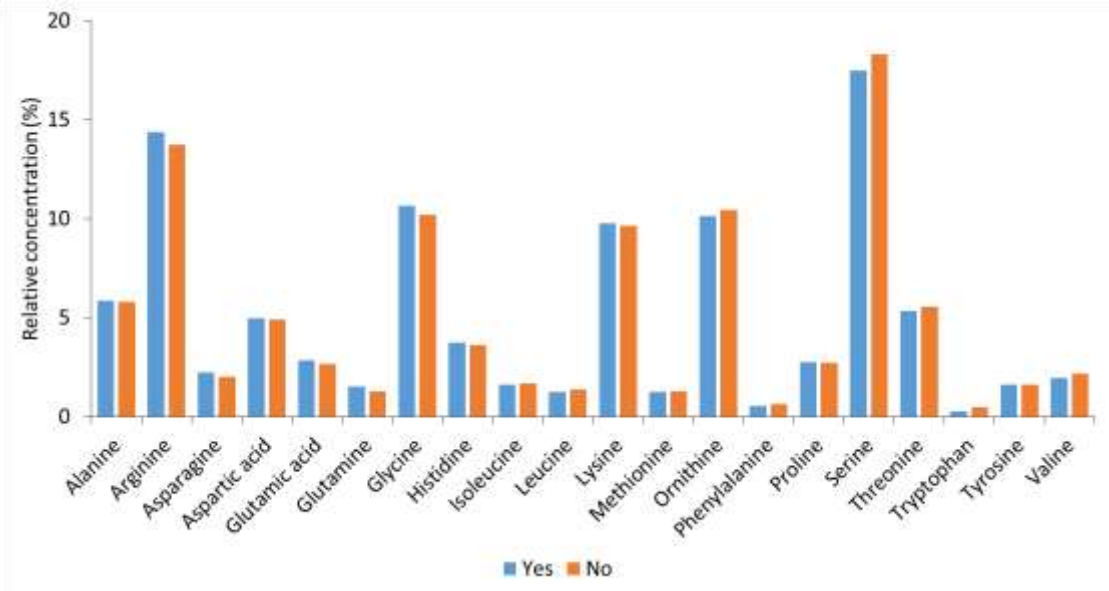


Figure 6.14 Relative, to the sum of total amino acid concentration, abundance of amino acids in donors who had or had not consumed food within one hour prior to fingermark deposition.

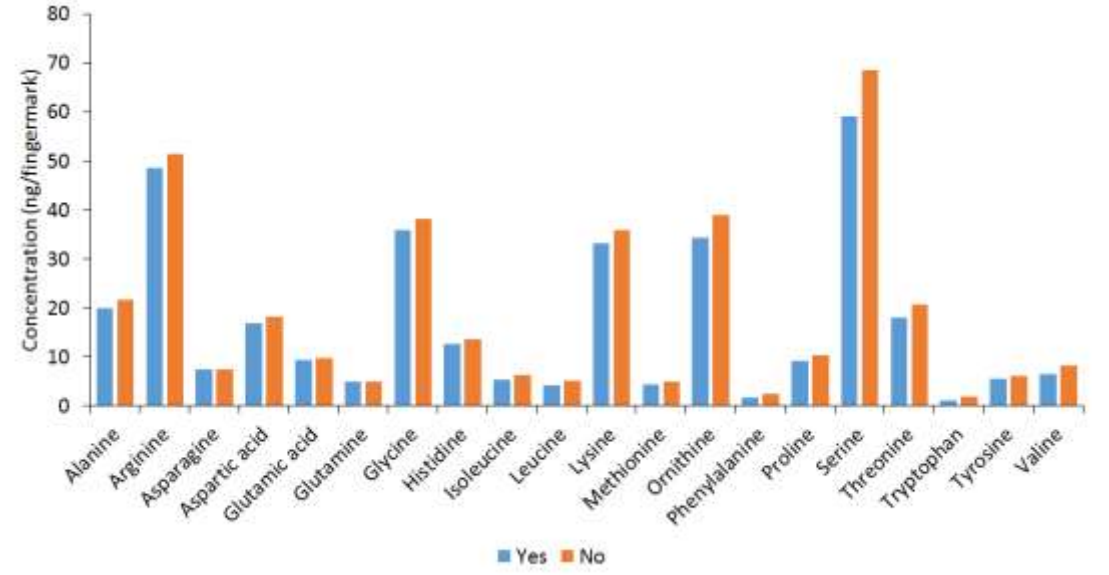


Figure 6.15 Absolute abundance of amino acids in donors who had or had not consumed food within one hour prior to fingermark deposition.

Arginine, glycine, serine, ornithine and histidine displayed the largest difference in relative amino acid abundance between donors who had used cosmetics within 12 hours prior to giving fingermark deposits to those who had not (Figure 6.16). Changes in these amino acids were also observed in the HPLC-UV-DAD results presented in Chapter 5. Glycine, serine and arginine showed the greatest difference in the absolute amino acid concentrations (Figure 6.17). However, in both cases these differences are minimal and the actual profiles are quite similar.

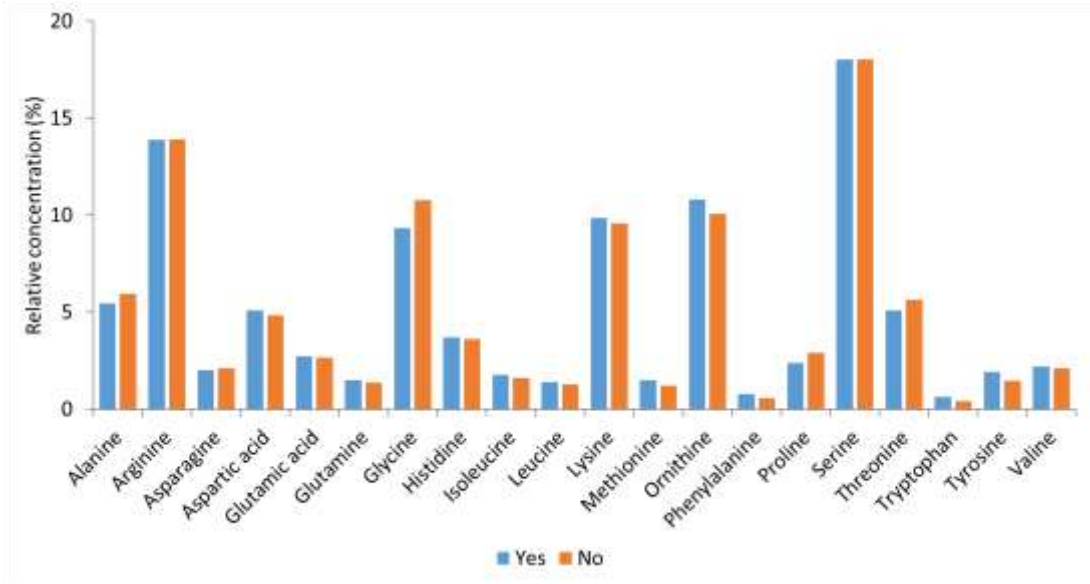


Figure 6.16 Relative abundance of amino acids, relative to the sum of total amino acid concentration, for donors who had and had not used cosmetics within 12 hours of giving fingermark deposits.

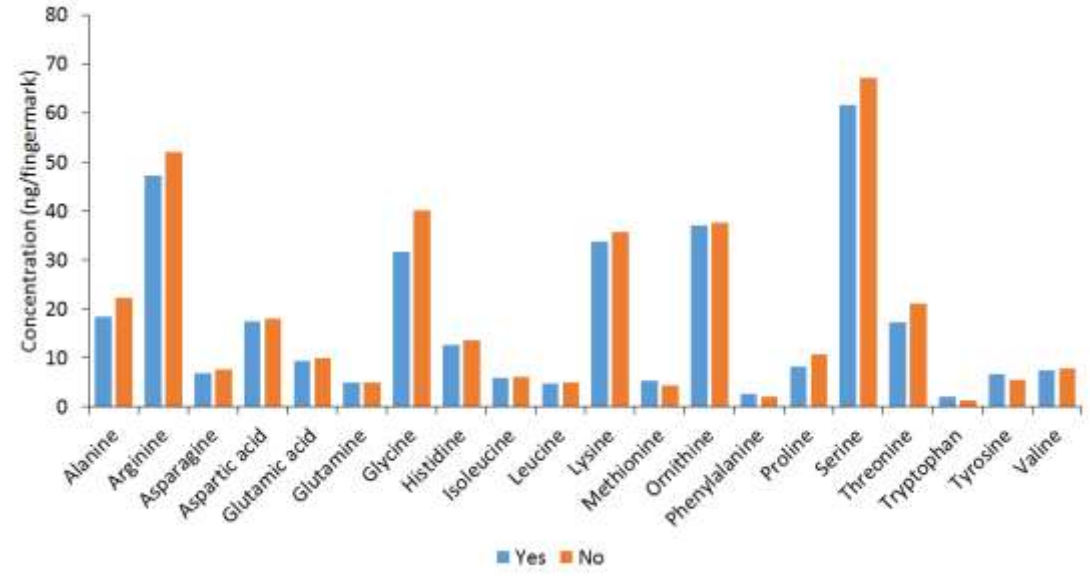


Figure 6.17 Absolute abundance of amino acids for donors who had and had not used cosmetics within 12 hours of giving fingermark deposits.

6.3.5 Statistical data evaluation

The same statistical approach which was used in Chapter 5 was applied to the LC-MS results. The Mann-Whitney U test was used to compare the medians of the absolute and relative concentrations of all samples to determine whether there are significant differences due to donor traits and habits. In this case, the 5 most abundant amino acids were serine, arginine, ornithine, glycine and lysine (in that order), which make up 62 % of the total amino acid concentration in a fingermark deposit. While the 5 most abundant amino acids found in the HPLC-UV-DAD studies made up a similar proportion (63 %), they were comprised of serine, glycine, ornithine, alanine, and aspartic acid. As the statistical outcomes are the same for either set of the most abundant amino acids, the discussions below will focus on the serine, glycine, ornithine, alanine, and aspartic acid results to offer consistency.

Prior to examinations of the donor traits and habits using the Mann-Whitney U test, Wilcoxon signed rank tests were used to investigate whether statistically significant differences could be observed between the relative amino acid profiles of the LC-MS and HPLC-UV-DAD results. Initially, a comparison of the two instruments was made as a function of the donor. In that case, all amino acid results for a donor were compared between the HPLC-UV-DAD and LC-MS instruments. This resulted in p and Z values of 0.660 and -0.530, respectively, indicating that no significant difference was found between the two instruments (Appendix D). The two instruments were then compared as a function of the amino acid, i.e. the median value of each amino acid from all donors was compared between the HPLC-UV-DAD and LC-MS instruments. Here, 15 out of 20 amino acids showed significant differences between the two methods (Appendix E). One possible explanation for this discrepancy could be due to the manner of the statistical approach. The Wilcoxon signed rank test works by ranking the amino acids from each donor and then comparing the classification of each value, in this case the identity of the individual amino acid is lost. A further explanation may be the large standard deviations which exist in this highly variable dataset. The chosen confidence interval (where p=0.05) is based on the fact that 95 % of the distribution lies within 1.96 standard deviations of the mean. Therefore, the data that is more than 1.96 standard

deviations away from the centroid is assumed to be different to the population mean. With datasets with very large standard deviations, such as this one, the confidence interval can encompass a very broad range of mean values without being violated.

6.3.5.1 Donor traits

The sex of the donor did not appear to affect the most abundant amino acids, where an average p value of 0.381 and a Z score of -1.032 was calculated for the relative abundance, and p = 0.115 and Z = -1.618 for the absolute concentration (Table 6.11). These results reflect the findings of the pilot and donor study in Chapter 4, as well as the results of Chapter 5. Out of 20 amino acids only tyrosine, glutamic acid, and asparagine were shown to be significantly different in the absolute concentrations (and leucine borderline with a p value of 0.055), and no amino acids were seen to be significantly different with male or female donors in relative concentration comparisons. The dissimilarity found in glutamic acid, asparagine and leucine was also reported for HPLC-UV-DAD results in Chapter 5.

Unlike the previous chapter, there was no significant difference (average p = 0.409, Z = -1.174) between donors over and under the age of 25 in the absolute concentration of the 5 most abundant amino acids, see Table 6.11. Only alanine and asparagine were significantly different, where alanine was found to be the one amino acid in the HPLC-UV-DAD study to not show any difference. When comparing the relative concentrations, the average p and Z values were 0.219 and -1.734, respectively. This means that there is no significant difference between donors over and under the age of 25. Alanine, arginine, asparagine, aspartic acid and glutamic acid were the only amino acids showing differences as a function of age of the donor and relative analyte abundance. In this investigation, changing the donor age to those under 15 and donors 20 or over did not significantly change the results. These findings are opposed to the conclusions drawn in Chapter 4 and 5.

Table 6.11 Statistical values gained from Mann-Whitney U tests, with the relative and absolute concentrations of amino acids given as a function of the independent variables.

Variable	Absolute concentration				Relative concentration			
	Sex p value	**Sex Z value**	**Age p value**	**Age Z value**	**Sex p value**	**Sex Z value**	**Age p value**	**Age Z value**
Alanine	0.089	-1.700	0.514	-0.652	0.536	-0.619	0.386	-0.866
Arginine	0.976	-0.029	0.044	-2.015	0.284	-1.071	0.271	-1.100
Asparagine	0.028	-2.192	0.553	-0.594	0.437	-0.776	0.408	-0.827
Aspartic acid	0.069	-1.818	0.280	-1.080	0.093	-1.681	0.005	-2.813
Glutamic acid	0.026	-2.231	0.763	-0.302	0.716	-0.364	0.477	-0.710
Glutamine	0.128	-1.523	0.793	-0.263	0.761	-0.305	0.633	-0.477
Glycine	0.234	-1.189	0.131	-1.509	0.914	-0.108	0.002	-3.085
Histidine	0.063	-1.860	0.915	-0.107	0.821	-0.226	0.188	-1.316
Isoleucine	0.184	-1.327	0.823	-0.224	0.644	-0.462	0.884	-0.146
Leucine	0.055	-1.916	0.930	-0.088	0.658	-0.442	0.255	-1.139
Lysine	0.097	-1.661	0.838	-0.204	0.178	-1.346	0.182	-1.333
Methionine	0.104	-1.624	0.693	-0.394	0.959	-0.051	0.976	-0.030
Ornithine	0.089	-1.700	0.386	-0.866	0.258	-1.130	0.239	-1.178
Phenylalanine	0.069	-1.818	0.113	-1.586	0.267	-1.111	0.001	-3.475
Proline	0.149	-1.445	0.326	-0.983	0.536	-0.619	0.216	-1.236
Serine	0.093	-1.681	0.763	-0.302	0.105	-1.622	0.465	-0.730
Threonine	0.154	-1.425	0.930	-0.088	0.961	-0.049	0.298	-1.041
Tryptophan	0.504	-0.668	0.004	-2.851	0.570	-0.568	0.004	-2.851
Tyrosine	0.010	-2.588	0.331	-0.972	0.129	-1.517	0.644	-0.461
Valine	0.109	-1.602	0.170	-1.372	0.914	-0.108	0.001	-3.377

6.3.5.2 Donor habits

In agreement with Chapter 5, donors who had or had not washed their hands prior to fingermark deposition were found not to give significantly different deposits as a function of their relative amino acid concentrations ($p = 0.168$, $Z = -1.625$), Table 6.11. However, the absolute abundance shows a significant difference as a function of washing hands ($p = 0.027$, $Z = -2.251$). As was observed in Figure 6.13 above, the concentrations of all amino acids were reduced after washing hands. Out of 20 amino acids, only tryptophan, tyrosine and valine were not found to be significantly different. These findings are in keeping with the pilot and donor study presented in Chapter 4.

No statistically significant dissimilarity was found between the absolute amino acid concentration from donors who had or had not recently consumed food ($p = 0.483$, $Z = -0.724$) or had applied cosmetics ($p = 0.627$, $Z = -0.495$), Table 6.12. This was in agreement with the relative abundance (food: $p = 0.604$, $Z = -0.557$; cosmetics: $p = 0.504$, $Z = -0.761$) and the results of the donor study in Chapter 4 and the HPLC-UV-DAD investigation in Chapter 5.

As in Chapter 5, the absolute and relative abundances of the amino acids extracted from latent fingermark deposits were further investigated using PCA. This chemometric approach was again applied in view of all donor variables, where it was found that no clustering occurred due to any trait or habit. A reduction in the number of amino acids to those that showed a significant difference in the Mann-Whitney U tests resulted in no apparent clustering. As before, this may be an effect of the significant standard deviation associated with each variable and amino acid, due to the large intra- and inter-donor variation.

Table 6.12 Statistical values gained from Mann-Whitney U tests, with the relative and absolute concentrations of amino acids given as an effect of the independent variables.

Variable	Absolute concentration						Relative concentration					
	Washing of hands p value	Washing of hands Z value	Food p value	Food Z value	Cosmetics p value	Cosmetics Z value	Washing of hands p value	Washing of hands Z value	Food p value	Food Z value	Cosmetics p value	Cosmetics Z value
Alanine	0.012	-2.498	0.533	-0.624	0.868	-0.166	0.121	-1.552	0.677	-0.416	0.677	-0.416
Arginine	0.033	-2.134	0.771	-0.291	0.803	-0.250	0.073	-1.795	0.852	-0.187	0.176	-1.352
Asparagine	0.112	-1.589	0.685	-0.406	0.934	-0.083	0.894	-0.133	0.892	-0.135	0.884	-0.146
Aspartic acid	0.039	-2.062	0.417	-0.811	0.429	-0.790	0.094	-1.673	0.533	-0.624	0.197	-1.289
Glutamic acid	0.029	-2.183	0.318	-0.998	0.967	-0.042	0.065	-1.843	0.417	-0.811	0.803	-0.250
Glutamine	0.013	-2.474	0.618	-0.499	0.724	-0.354	0.160	-1.407	0.176	-1.352	0.950	-0.062
Glycine	0.044	-2.013	0.739	-0.333	0.603	-0.520	0.561	-0.582	0.771	-0.291	0.145	-1.456
Histidine	0.017	-2.380	0.917	-0.104	0.917	-0.104	0.610	-0.510	0.328	-0.979	0.190	-1.312
Isoleucine	0.009	-2.621	0.835	-0.208	0.603	-0.520	0.145	-1.456	0.693	-0.395	0.771	-0.291
Leucine	0.026	-2.231	0.771	-0.291	0.739	-0.333	0.410	-0.825	0.950	-0.062	0.244	-1.165
Lysine	0.005	-2.789	0.371	-0.894	0.739	-0.333	0.133	-1.504	0.724	-0.354	0.339	-0.957
Methionine	0.151	-1.437	0.560	-0.584	0.462	-0.735	0.860	-0.176	0.966	-0.043	0.243	-1.167
Ornithine	0.026	-2.231	0.454	-0.749	0.560	-0.582	0.021	-2.304	0.819	-0.229	0.868	-0.166
Phenylalanine	0.007	-2.692	0.096	-1.664	0.253	-1.144	0.008	-2.644	0.017	-2.392	0.015	-2.433
Proline	0.013	-2.474	0.739	-0.333	0.771	-0.291	0.033	-2.134	0.835	-0.208	0.787	-0.270
Serine	0.014	-2.450	0.270	-1.102	0.677	-0.416	0.044	-2.013	0.220	-1.227	0.632	-0.478
Threonine	0.014	-2.450	0.220	-1.227	0.647	-0.458	0.174	-1.358	0.244	-1.165	0.124	-1.539
Tryptophan	0.004	-2.901	0.024	-2.256	0.084	-1.729	0.015	-2.434	0.011	-2.551	0.177	-1.349
Tyrosine	0.405	-0.832	0.817	-0.231	0.413	-0.818	0.732	-0.343	0.615	-0.504	0.476	-0.713
Valine	0.013	-2.474	0.170	-1.373	0.328	-0.977	0.077	-1.771	0.220	-1.227	0.014	-2.454

6.3.6 Simulants

Further to the analysis of a commercially sourced amino acid fingermark simulant using HPLC-UV-DAD in Chapter 5, the Orbitrap confirmed the previous findings. As can be seen in Table 6.13, the absolute and relative concentrations of the amino acids are quite different in most cases between a real fingermark deposit and the simulant sample. The total abundance of amino acids was also 25 % higher in the simulated samples, which has been observed anecdotally in the increased development of deposits after the treatment with amino acid sensitive reagents.

Table 6.13 Abundance of amino acids, both absolute values and relative to the sum of total amino acid concentration, of the average of real latent fingermarks versus a simulant sample.

Amino acid	Absolute concentration (ng/deposit)		Relative concentration (%)	
	Simulant	Fingermark average	Simulant	Fingermark average
Alanine	12.3	21.0	2.5	4.8
Arginine	109.5	50.4	22.5	17.6
Asparagine	29.7	7.5	6.1	2.0
Aspartic acid	6.9	17.7	1.4	4.2
Glutamic acid	12.6	9.6	2.6	3.0
Glutamine	15.3	5.1	3.1	1.7
Glycine	25.5	37.5	5.2	9.3
Histidine	40.8	13.2	8.4	3.6
Isoleucine	10.5	6.0	2.2	1.7
Leucine	7.8	4.8	1.6	1.4
Lysine	73.8	35.1	15.2	11.9
Methionine	18.3	4.8	3.8	1.4
Ornithine	56.7	37.5	11.6	12.3
Phenylalanine	3.6	2.4	0.7	0.6
Proline	8.4	9.9	1.7	2.3
Serine	8.4	65.4	1.7	13.8
Threonine	13.8	19.8	2.8	4.5
Tryptophan	6.9	1.5	1.4	0.4
Tyrosine	16.2	5.7	3.3	1.7
Valine	9.9	7.8	2.0	1.9
Total	486.9	362.7	-	-

A further example is the very high concentration of cysteine. While this analyte could not be quantified, the peak area was found to be much greater than serine, which is also in agreement with the chromatogram shown in Figure 5.23, Chapter 5. However, the main differences lie within the additional compounds that were not found in fingermark deposits but were observed in simulant samples. For example, Figure 6.18 shows that in addition to the deuterated alanine standard (8.77 min), a second signal with a *m/z* ratio of 93 is displayed (10.39 min). In this case, the exact *m/z* was 93.0546, which was attributed to glycerol ($C_3H_8O_3$). It is the presence of these exogenous compounds that may affect the efficacy of development reagents and therefore lead to forming wrong conclusions based on the results.

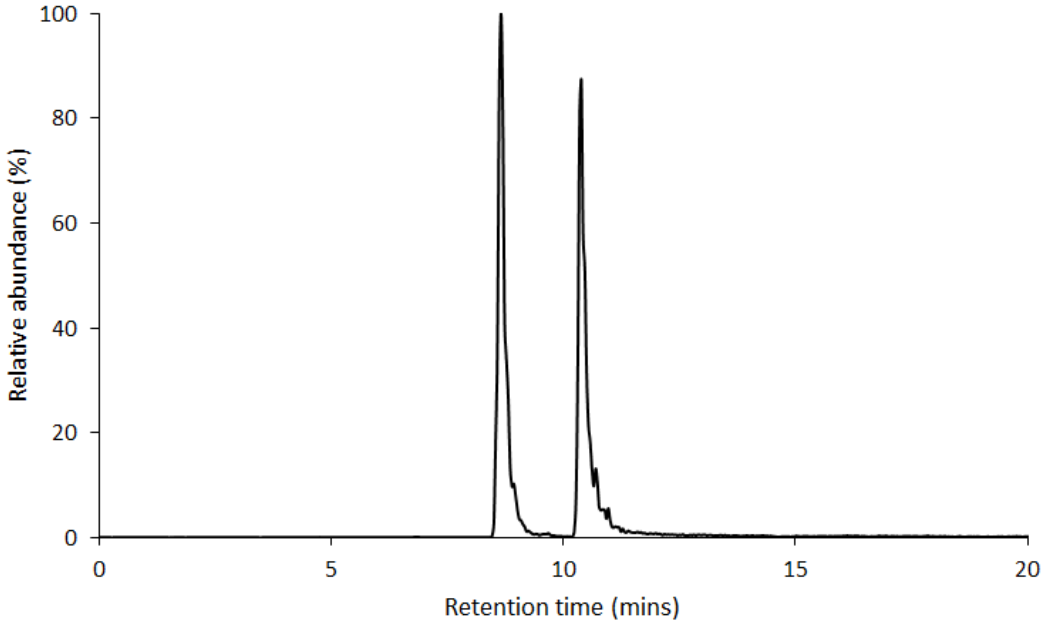


Figure 6.18 Sample chromatogram of the deuterated alanine standard (8.77 min) and glycerol (10.39 min) found in an extracted simulant sample using the Orbitrap. Samples were analysed using a Phenomenex Gemini C18 column (250 mm×3 mm i.d., 3 µm particle size) and an Orbitrap mass spectrometer operated in the scan mode.

6.4 Conclusions

An existing method used by Swann *et al.* for the determination of amino acid content in decaying pig tissue, and updated by How *et al.* for a larger number of amino acids in a water matrix, was used as a starting point for this work on a triple quadrupole LC-MS [214, 252]. This method was expanded upon to include l-ornithine in the analysis, which could be separated from asparagine despite a very similar molecular ion due to the difference in fragmentation pattern and 2 minute quicker elution time. Extraction of fingermark deposits from filter paper circles, with a 2.5 cm diameter, using a 50 % (v:v) methanol:water soak for one hour was seen as a quick, reproducible and efficient manner of preparing the samples for amino acid detection.

No matrix effects for fingermark extracts were observed with the current method, which was also revised for increased sensitivity. A working detection method for the analysis of 20 amino acids was found for an Orbitrap based LC-MS system, where a combination of the existing triple quadrupole LC conditions were matched with the scan capabilities, rather than MS/MS, of the Orbitrap instrument.

Fingermark samples from 50 donors were analysed, where exhibits from 3 fingers for each donor were collected. Serine was found to be the predominant amino acid, followed by arginine, ornithine, glycine, lysine and alanine (in that order). The average concentration of each amino acid per fingermark was found to be between 1.5 ng (tryptophan) and 65.4 ng (serine). The sum of the average amino acid concentrations was shown to be 363 ng per fingermark, but ranged from 66 to 1371 ng per fingermark depending on the donor. The inter-donor studies can be split into those which concern the donor traits (i.e. age and sex) and donor habits (such as recent food consumption). Comparing the absolute and relative concentrations of serine, glycine, ornithine, alanine, and aspartic acid, it was found that there was no significant difference due to food consumption, use of cosmetics, age of the donor or biological sex. The absolute concentration deposited by donors who had and had not washed their hands within one hour of fingermark deposition were statistically dissimilar in 17 out of 20 amino acids, and in all analytes increased abundances were recorded from donors who had not washed their hands. Aside from the results for the age of the donors and the washing of hands (in terms of absolute concentrations), all

outcomes reflect the findings of Chapter 5. The age of the donor was also the one variable which was not in agreement with the results of the donor study in Chapter 4.

PCA of the data showed that no significant clustering occurred for any donor trait or habit. Limiting the number of amino acids to those that were found to be significantly different between variables using the Mann-Whitney U test resulted in no further discrimination.

Differences in the results of the HPLC-UV-DAD (Chapter 5) and LC-MS instruments highlight the difficulty in quantifying these analytes at very low concentrations after extraction from a complex matrix. Further method optimisation and a larger donor pool may offer more comparable outcomes across all the investigated variables.

Comparison of the chromatograms of amino acid simulant samples show significant differences to those of real latent fingermark deposits, reinforcing the findings by Zadnik *et al.* [151]. Although the differences in amino acid concentrations are within one order of magnitude, the actual profile of amino acids is different. More importantly, analytes which were below the limit of detection in latent fingermark residue were found to be significant in the simulant samples. Due to the chemical differences, caution should be used when treating simulants as fingermark standards.

Chapter 7
Conclusions and suggestions for future work

7.1 Conclusions

The aims of this thesis were to develop novel fingermark reagents and to investigate the amino acid content of latent fingermarks to aid in the systematic development of new and improved fingermark detection methods.

A new *p*-dimethylaminobenzaldehyde (DMAB) based reagent yielded fingermark development on paper surfaces that are both coloured and photoluminescent. The wet contact method was effective on non-fragile porous substrates such as white copy paper and various other substrates. Fingermarks deposited on thermal paper and other porous substrates were visualised using the dry contact DMAB approach. These methods were suitable for use on strong fingermark deposits; however, they did not offer the same level of development as ninhydrin or 1,2-indanedione/zinc chloride (IND/$ZnCl_2$) on very weak impressions.

A new and improved formulation of *p*-dimethylaminocinnamaldehyde (DMAC), using a low polarity solvent and heat-free treatment, allowed the visualisation of fingermarks on a variety of substrates, including thermal paper, without modification to the formulation. In comparison to previously published formulations, the new approach afforded a more rapid and sensitive detection of latent fingermarks. Similar levels of development are afforded to IND/$ZnCl_2$ on all but very weak deposits.

Wilcoxon signed rank tests and intraclass correlation coefficients demonstrated that the British Home Office's grading scale, which was used to evaluate the reagent response, is a consistent and reliable fingermark grading method. Only 0.5 % of the total grades given to IND/$ZnCl_2$ treated fingermarks returned a score of 0 and 64.6 % of all grades given were a 3 or 4 (where 4 is the highest score).

Mann-Whitney U and Wilcoxon signed rank tests demonstrated that the response of the amino acid sensitive reagent IND/$ZnCl_2$ was significantly affected by the donor traits/habits within a population of 250 individuals. The grades of fingermarks developed within 3 days were shown to vary significantly as an effect of the age of the donor and the washing of hands prior to deposition. Donors who did not wash their hands the hour prior to deposition, and/or were below the age of 25, were more likely to offer higher grades. No significant variation between the fingermark grade

and food consumption, sex of the donor or recent use of cosmetics was observed with fresh fingermarks.

Liquid chromatography mass spectrometry (LC-MS) and high performance liquid chromatography coupled with an ultraviolet - diode array detector (HPLC-UV-DAD) instruments were used to establish the profile of 21 amino acids within a population of 50 donors. Serine was the most abundant amino acid in all samples, and the average amino acid concentration was shown to be 520 ng per fingermark. In comparing the absolute and relative concentrations of the 5 most abundant amino acids (serine, glycine, ornithine, alanine, and aspartic acid), it was found that there was no significant difference due to consumption of food, use of cosmetics, or the donor's biological sex.

HPLC-UV-DAD results showed that the absolute amino acid concentration within donors over and under the age of 25 was statistically dissimilar in the 5 most abundant amino acids. The absolute amino acid concentration within donors who had and had not washed their hands within one hour of fingermark deposition were shown to be statistically dissimilar in the 17 out of 20 amino acids analysed using LC-MS. In all analytes increased abundances were recorded from donors who had not washed their hands prior to sample deposition.

The outcomes of this study have demonstrated the potential of two novel fingermark development reagents and that these reagents target amino acids. They have also indicated potential future studies in this field.

7.2 Future work

Further studies are desirable to more fully investigate the operational potential of DMAB and DMAC for latent fingermark detection. These include studies into a wider range of substrates and the effect of including other components, such as metal salts, in the formulation or as a post treatment. In addition there is a need to synthesise and isolate the photoluminescent imine reaction products. Once the properties and reaction mechanisms of these are better understood, there is the potential to rationally design and synthesise analogues of DMAB/DMAC that provide improved performance as fingermark detection reagents. As DMAC gives

more intense colouration and yields better quality fingermark detail, it can be inferred that more highly conjugated push-pull systems may enhance on this trend.

The confidence in the donor trait outcomes (Chapter 4) could be increased by expanding the donor population to incorporate more donors in all age groups. An increase in the population size may give more weighting to the outcomes of the studies where fingermark reagents are used to facilitate the evaluation of donor traits. Using two different amino acid sensitive reagents could give further confidence to the conclusions drawn if their respective results are in agreement. This would minimise any effect that may be caused by any preferential reaction of the reagent. For this reason, the reagents should also not be analogues (e.g. 1,2-indanedione, ninhydrin or 1,8-diazafluoren-9-one) but rather should be structurally unrelated (e.g. 1,2-indanedione and DMAC). A further point of interest would be use of very specific reagents, such as immunolabeling, in conjunction with LC studies which could indicate whether it is the amino acid abundance or profile that affects reagent performance. While recent food consumption was not found to be statistically dissimilar in these studies, longer timeframes (for example 6 or 12 months) may potentially reveal that treated and untreated amino acids degrade faster after the consumption of food. These longer timeframes may also show whether other donor traits and habits (such as recent use of cosmetics or washing of hands) can affect a fingermark's lifetime. The comparison between the grading results from fingerprint identification experts and normal fingermark researchers would also be interesting to observe in further work, where the difference in experience and a shift in the attention from overall quality to ridge detail may change the outcomes.

Although the alternative LED light source was found to offer reduced illumination to the Polilight, it may be useful in teaching or remote environments where portability is an issue and the expense of the Polilight cannot be justified. The use of the LED light also needs to be further investigated for application with a range of different reagents, such as IND/$ZnCl_2$. Handheld torches from the same manufacturer as the LED light (Cree, USA) are very cheap to buy and are available in a range of colours. These could be further explored to see whether they are useful as a cheap alternative to forensic light sources, and if they can be used for teaching purposes.

Differences in the results of the HPLC-UV-DAD (Chapter 5) and LC-MS (Chapter 6) instruments highlight the difficulty in quantifying amino acids at very low concentrations after extraction from a complex matrix. While LC-MS is the more sensitive and specific technique, more researchers have access to the simpler and cheaper HPLC-UV-DAD instrumentation, which in addition to allowing the comparison of the results, facilitates the possibility of future and repeat experiments by external institutions. Improvements to the sensitivity of the liquid chromatography methods would benefit the amino acid quantification of very 'weak' donors, as it was found that in these cases detector response approached the lower limit of quantification. This could be achieved by changing the chromatographic conditions, using different derivatisation agents for the HLPC-UV method and to incorporate different instruments into the study. While not available for this project, HPLC coupled to fluorescence detectors is known to be more sensitive than UV [64]. As per de Puit's method, amino acids could also be derivatised for the LC-MS method to improve the limit of detection and the separation [35]; however, this would complicate the chromatographic method. Further instruments, such as matrix assisted laser desorption/ionisation [91], capillary electrophoresis mass spectrometry [36, 85] and desorption electrospray ionisation [254], have been used in the past for amino acid analysis and could provide complementary information to the two methods used in this study.

While the focus of this PhD project was on the amino acid content of latent fingermark deposits, a range of further endogenous material can be analysed by using the LC-MS instrument. Exploratory investigations could show further chemical species that are readily found, quantifiable and possibly indicative of donor traits but have been ignored in this more restricted project. Orbitrap instruments in particular are very suitable for discovery work, where mass resolution, high mass accuracy and multiple fragmentation stages aid the identification of unknown analytes in the samples [233]. By scanning the entire spectrum (in the case of this study limited to a *m/z* range of 50-250), the Orbitrap instrument has already recorded further compounds in addition to the 21 amino acids for 50 donors, although it was outside of the scope of this project to further investigate additional endogenous material.

The HPLC-UV-DAD and LC-MS studies are preliminary in nature, where 50 donors are ultimately not a large enough population to derive any concrete trends. In increasing the size of the donor pool, more confidence can be placed into the outcomes of the study. However, it was found that gaining access to, and co-operation with, a large number of individuals is a very arduous and time consuming task, where more targeted studies (e.g. for a particular donor trait) may be more realistically achievable within the constraints of a project.

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Appendices

Appendix A Collection questionnaire for fingermark donors.

Donor number:

Age (years):

Gender:

Date of collection:

Washing of hands (< 1hr):

Y - N

Food handling (< 1hr):

Y - N

Washing of hands since handling food:

Y - N

Recent use of cosmetics/skin care products (within 12hrs):

Y - N

Recent use of cosmetics/skin care products (within 24hrs):

Y - N

Recent handling of any other greasy/dirty substances within 12 hrs (please describe if yes):

Y - N

_____

Appendix B Relative standard deviations of the various extraction methods for each amino acid (%).

	Extraction efficiency (%)							
	30 % (v:v) methanol:water				50 % (v:v) methanol:water			
Amino acid	**Agitation (5 mins)**	**Agitation (10 mins)**	**No agitation (30 mins)**	**No agitation (60 mins)**	**Agitation (5 mins)**	**Agitation (10 mins)**	**No agitation (30 mins)**	**No agitation (60 mins)**
Aspartic acid	23.26	32.78	2.75	2.33	29.11	3.46	5.26	6.95
Glutamate	25.05	33.47	2.93	0.94	33.00	3.25	4.80	2.46
Asparagine	29.89	33.98	1.31	1.08	42.52	3.03	2.91	1.75
Serine	22.61	33.60	1.29	1.37	34.60	2.49	3.37	1.61
Glutamine	23.94	33.78	1.44	1.09	35.34	3.48	3.26	1.36
Glycine	6.92	33.80	1.35	1.89	10.96	1.82	3.55	1.28
Histidine	2.37	30.34	12.19	2.43	11.13	9.59	12.81	5.72
Arginine	18.09	31.16	3.36	1.11	27.49	3.95	1.53	2.31
Threonine	21.95	30.73	2.93	2.49	31.58	3.36	3.89	1.20
Alanine	16.32	39.69	4.64	1.82	22.79	4.20	5.08	2.80
Proline	4.20	31.99	1.24	1.48	2.26	2.32	3.56	1.10
Cysteine (minor)	11.54	28.46	4.63	3.80	19.50	3.26	5.44	0.64
Aminbutyric acid (IS)	2.53	27.35	1.95	0.38	5.14	2.71	1.95	0.79
Tyrosine	7.97	31.76	4.84	1.70	17.49	3.77	5.14	3.02
Valine	4.09	34.03	4.30	0.38	13.02	3.47	5.46	1.74
Methionine	34.83	39.46	19.80	18.22	10.52	7.35	3.49	12.07
Cysteine (major)	4.24	35.83	5.99	1.39	8.19	5.01	5.15	4.61
Isoleucine	4.49	35.87	7.59	2.66	11.92	4.18	6.88	4.34
Leucine	4.30	36.14	8.41	5.37	11.67	4.51	6.51	2.20
Norleucine (IS)	5.34	31.95	6.69	1.15	1.00	2.62	3.69	2.45
Phenylalanine	8.30	33.61	7.18	0.70	17.07	4.17	2.38	4.47
Ornithine	7.87	2.58	9.80	4.81	3.28	2.83	1.16	3.29
Tryptophan	5.86	28.33	8.61	1.29	10.12	3.06	3.10	3.46
Lysine	23.26	32.78	2.75	2.33	29.11	3.46	5.26	6.95
Average	13.30	31.81	5.33	2.59	18.28	3.81	4.40	3.27

Appendix C Relative standard deviations of the various extraction methods for each amino acid (%).

	Extraction efficiency (%)							
	30 % (v:v) methanol:water				50 % (v:v) methanol:water			
Amino acid	**Agitation (5 mins)**	**Agitation (10 mins)**	**No agitation (30 mins)**	**No agitation (60 mins)**	**Agitation (5 mins)**	**Agitation (10 mins)**	**No agitation (30 mins)**	**No agitation (60 mins)**
Alanine	5.89	6.30	13.34	8.18	11.10	10.12	13.54	10.33
Alanine-d_3	7.09	28.33	11.36	40.23	14.35	23.91	5.57	4.01
Arginine	5.67	16.82	3.98	6.57	6.34	12.49	6.73	2.70
Asparagine	6.25	1.60	7.03	7.95	6.02	3.33	6.73	4.03
Aspartic acid	4.92	5.19	2.75	5.34	3.89	4.05	7.02	5.39
Cysteine	48.74	55.42	13.97	35.50	36.38	31.09	40.91	13.23
Glutamic acid	3.69	2.26	4.45	3.97	2.80	3.44	4.30	4.75
Glutamic-d_3 acid	6.99	21.13	7.73	41.11	12.06	23.67	42.73	6.35
Glutamine	4.06	3.28	3.82	1.49	3.39	2.92	5.62	5.48
Glycine	4.34	8.02	1.14	3.41	4.58	4.61	6.89	4.70
Glycine-d_2	8.75	20.14	39.54	13.15	20.26	28.19	11.89	9.84
Histidine	12.78	17.18	3.64	8.07	5.76	6.76	4.16	2.62
Isoleucine	6.24	3.35	21.87	44.40	2.67	2.31	10.88	2.53
Leucine	6.24	3.35	21.87	44.40	2.67	2.31	10.88	2.53
Leucine-d_3	5.91	21.25	7.71	41.07	10.96	21.96	42.93	2.83
Lysine	4.08	3.27	3.41	1.48	3.41	2.93	5.65	5.51
Methionine	4.71	11.98	6.68	4.62	3.64		1.77	1.97
Ornithine	1.86	21.13	2.75	11.24	5.94	22.45	9.12	3.41
Phenylalanine	25.30	37.70	13.40	10.98	18.20	7.73	15.69	7.68
Phenyl-d_5-alanine-d_3	10.43	21.52	40.27	39.68	15.30	25.42	8.10	4.51
Proline	4.79	2.85	5.49	3.11	4.00	2.21	4.59	1.75
Serine	5.33	1.67	4.06	3.64	4.92	3.20	3.37	4.67
Threonine	7.12	2.36	2.97	3.42	2.73	6.27	8.83	4.64
Tryptophan	5.26	10.85	3.64	4.32	8.90	18.38	16.22	5.58
Tyrosine	4.51	4.47	5.63	1.45	3.59	3.85	2.96	3.18
Valine	3.16	22.27	14.49	3.29	8.82	26.15	11.05	3.20
Average	8.24	13.60	10.27	15.08	8.56	11.99	11.85	4.90

Appendix D Results of Wilcoxon sign rank test comparing the HPLC-UV-DAD and LC-MS instruments as a function of the donor.

Donor	p value	Z value	Donor	p value	Z value
Donor 1	0.881	-0.149	Donor 26	0.586	-0.544
Donor 2	0.573	-0.563	Donor 27	0.970	-0.037
Donor 3	0.073	-1.792	Donor 28	0.940	-0.075
Donor 4	0.084	-1.730	Donor 29	0.940	-0.075
Donor 5	0.396	-0.849	Donor 30	0.709	-0.373
Donor 6	0.057	-1.904	Donor 31	0.872	-0.161
Donor 7	0.086	-1.717	Donor 32	0.575	-0.560
Donor 8	0.478	-0.709	Donor 33	0.433	-0.784
Donor 9	0.023	-2.277	Donor 34	0.575	-0.560
Donor 10	0.601	-0.523	Donor 35	0.881	-0.149
Donor 11	0.881	-0.149	Donor 36	0.881	-0.149
Donor 12	0.881	-0.149	Donor 37	0.794	-0.261
Donor 13	0.048	-1.979	Donor 38	0.478	-0.709
Donor 14	0.681	-0.411	Donor 39	0.940	-0.075
Donor 15	1.000	0.000	Donor 40	0.627	-0.485
Donor 16	0.970	-0.037	Donor 41	0.911	-0.112
Donor 17	0.737	-0.336	Donor 42	0.765	-0.299
Donor 18	0.823	-0.224	Donor 43	0.823	-0.224
Donor 19	0.748	-0.322	Donor 44	0.911	-0.112
Donor 20	0.970	-0.037	Donor 45	0.601	-0.523
Donor 21	0.970	-0.037	Donor 46	0.408	-0.827
Donor 22	0.709	-0.373	Donor 47	0.841	-0.201
Donor 23	0.911	-0.112	Donor 48	0.520	-0.644
Donor 24	0.737	-0.336	Donor 49	0.234	-1.189
Donor 25	0.911	-0.112	Donor 50	0.575	-0.560
Average	*0.660*	*-0.530*	*Average*	*0.660*	*-0.530*

Appendix E Results of Wilcoxon sign rank test comparing the HPLC-UV-DAD and LC-MS instruments as a function of the amino acid.

Amino Acid	**p value**	**Z value**
Alanine	0.000	-5.150
Arginine	0.000	-6.023
Asparagine	0.000	-6.021
Aspartic acid	0.005	-2.814
Glutamic acid	0.000	-4.986
Glutamine	0.000	-6.033
Glycine	0.000	-4.571
Histidine	0.680	-0.413
Isoleucine	0.001	-3.344
Leucine	0.675	-0.420
Lysine	0.000	-6.093
Methionine	0.000	-4.032
Ornithine	0.000	-5.362
Phenylalanine	0.000	-4.272
Proline	0.000	-3.982
Serine	0.000	-6.135
Threonine	0.851	-0.188
Tryptophan	0.000	-5.681
Tyrosine	0.096	-1.665
Valine	0.988	-0.014
Average	*0.165*	*-3.860*